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UNIVERSITY OF ALBERTA

Canola Seedling Blight in Alberta: pathogens, involvement of *Pythium* spp. and biological control of *Rhizoctonia solani*

by

Albert I. Calman

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Master of Science

IN

Plant Pathology

Department of Plant Science

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SIGNED: Albert Calman

PERMANENT ADDRESS:

#414 9950-90A Avenue
Edmonton, Alberta
T6E 5A4

DATED: 15 October, 1990

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Canola Seedling Blight in Alberta: pathogens, involvement of Pythium spp. and biological control of Rhizoctonia solani, submitted by Albert I. Calman in partial fulfilment of the requirements for the degree of Master of Science in Plant Pathology.

Dr. J. P. Tewari (Supervisor)

Dr. C. Hiruki

Dr. N. R. Knowles

Dr. G. R. Webster

Date: 15 October, 1990

To

the memory of my grandmother, Leah Steinberg. *She was a very hard-working woman, who loved her husband, three daughters and sons-in-laws and all her grandchildren. She loved life, people and all nature.*

ABSTRACT

Canola seedling blight (CSB), an important disease resulting in serious reductions of canola (Brassica campestris L. and B. napus L.) seedling stands in Western Canada, was widespread in Alberta fields. Laboratory and growth cabinet controlled condition studies were undertaken to identify the fungi causing CSB in Alberta and to determine whether Trichoderma spp. can control CSB. Fungi were isolated from seedlings with CSB symptoms collected from 73 fields from throughout Alberta and additional Pythium spp. were isolated from canola seed placed into 24 collected field soils. Trichoderma spp. were applied onto seed and as soil amendments to control CSB in a Rhizoctonia solani Kuhn-infested soil. Brassica campestris cvs. Candle or Tobin were used in all experiments. Data were collected for both emergence and presence of symptoms on seedlings as measures of pre- and post-emergence damping-off, respectively, the two aspects of CSB.

Pathogens causing CSB included R. solani and F. avenaceum (Fries) Saccardo both isolated from field seedlings; P. pythioides (Roze and Cornu) Ramsbottom, P. ultimum Trow and Pythium sp. Group G Van der Plaats Niterink all isolated from colonized canola seed; and P. paroecandrum Drechsler and P. sylvaticum Campbell and Hendrix isolated from both field seedlings and colonized seed. Rhizoctonia solani and Pythium spp. were the most and least frequently isolated fungi, respectively, from field seedlings. Distinctive dot lesions on seedling cotyledons resulted from infection by Pythium spp. Observations of agar petri plates showed extensive coiling of hyphae of T. harzianum Rifai around hyphae of R. solani. Hyphae of R. solani were degraded in the presence of T. harzianum. Emergence of cv. Tobin was increased by 39.2% in R. solani-infested soil following the addition of a T. harzianum-wheat bran-chitin amendment. Percentage of cv. Tobin seedlings with CSB infection symptoms was decreased by 46.4% in R. solani-infested soil following the addition of this T. harzianum-wheat bran-chitin amendment.

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I. Introduction

A. Canola

Brassica spp. are grown worldwide, with two important products being an edible vegetable oil and a protein-rich meal. A 1983 report indicated on a worldwide basis, the planting of Brassica spp. oilseed crops on 1.1 million hectares of agricultural land, providing over 8% of the world's edible vegetable oil. In temperate regions, B. campestris L. (Polish or turnip rape) and B. napus L. (Argentine or oilseed rape) predominate for oilseed production (11).

Canola, a trademark of the Canola Council of Canada, refers to cultivars containing oil that has less than 5% erucic acid, and meal containing less than 3 mg glucosinolate per g moisture-free and oil-free meal (6,23).

In 1989, canola was planted on some 2.9 million hectares in Western Canada, with 1.1 million hectares being in Alberta. Percentage of 1989 acreage comprising of B. campestris and B. napus cultivars in Western Canada was 43.6% and 55.9%, respectively; and in Alberta it was 67.9% and 31.2%, respectively (7).

In this thesis, canola refers to both canola and rapeseed cultivars.

B. Diseases of canola seedlings

Canola seedlings are subject to a number of fungal diseases including blackleg caused by Leptosphaeria maculans (Desm.) Ces and de Not.; white rust caused by Albugo candida (Pers.) Ktze.; downy mildew caused by Peronospora parasitica Fr.; black spot caused by Alternaria brassicae (Berk.) Sacc. and A. raphani Groves and Skolko; and seedling blight caused by Rhizoctonia solani Kuhn, Fusarium spp., and Pythium spp. (6, 38).

C. Canola seedling blight

Canola seedling blight (CSB) occurs throughout the world and has increased in importance in Western Canada with the increased cultivation of this crop. Large losses of canola due to seedling blight, damping-off and brown girdling root rot have been reported in the Peace River region of Alberta (40).

Canola seedling blight consists of pre- and post-emergence damping-off. Pre-emergence damping-off referring to death of seed and/or seedlings before emergence, includes symptoms of seed being soft and/or discolored and seedlings having darkened water-soaked regions. Post-emergence damping-off referring to seedling death after emergence, includes symptoms of emerged seedlings with constrictions, discoloration and/or lesions (1).

Rhizoctonia solani has been reported to be the most frequently isolated fungus from and the principle pathogen causing CSB and root rot of adult canola plants (16,40). This fungus was shown to be highly virulent to canola seedlings, resulting in reduced emergence, wire-stem symptoms on hypocotyls and lesions on roots of emerged seedlings (22).

D. Rhizoctonia solani

Rhizoctonia solani was first observed on diseased potato tubers in 1858 (33). The perfect state of R. solani is a basidiomycete, Thanatephorus cucumeris (Frank) Donk. Rhizoctonia solani, a soil-borne fungus with a very widespread geographical distribution, exists as both a saprophyte and a parasite. As a pathogen, it is destructive on a great number of plants causing seed decay, damping-off, stem cankers, root rots, fruit decay and foliage diseases.

A number of distinctive characteristics of R. solani include rapidly growing pale to dark brown mycelium; branching at nearly right angles to main hyphae; constriction of, and presence of a septum in branch hypha near the point of origin; multinucleate hyphal

cells; presence of prominent dolipore septal pore apparati within hyphae; and production of sclerotia.

Isolates of R. solani have been seperated into anastomosis groupings (AG). This AG designation was first proposed by Schultz in 1937. A 1982 report listed seven AG's, with two of these each having two subgroups (2). Isolates belonging to the same AG, undergo fusion of hyphae and a killing reaction (death of hyphal cells adjacent to fused hyphae) when paired together. The existence of separate anastomosis groupings is based on the fact that R. solani is not a single species but is instead a collection of genetically isolated noninterbreeding populations (2).

In general, each AG of R. solani parasitizes a specific group of plants (2). Rhizoctonia solani AG 1 infects many plant species, causing seed and hypocotyl rot and aerial and web blights. Rhizoctonia solani AG 2 parasitizes cruciferous plants including Brassica spp., and results in seed rot and damping-off, root lesions and crown rot. This AG has been further divided into AG 2-1 and AG 2-2 which both infect canola (16). Studies from Western Canada report AG 2-1 to be more frequently isolated than AG 2-2 from canola with root and stem diseases (16,22,40). The AG 3 group mainly attacks potato plants, causing stem cankers, stolon lesions and black sclerotia on tubers. Rhizoctonia solani AG 4 infects a wide variety of plant species including beans, cotton, alfalfa and sugarbeet. This AG results in seed and root rot, lesions and rot of hypocotyls, stem lesions, crown rot and aerial blights.

i) Saprophytic growth of R. solani

In soil, R. solani exists saprophytically as mycelium and as sclerotia. It is known to be an early colonizer of buried plant tissues. Initial growth of R. solani has been shown to require a 'food base', with later growth being supported by available nutrients in the soil (3,33).

Several aspects of the saprophytic nature of R. solani in soil were reported in 1943 (3). Rhizoctonia solani was shown to maintain saprophytic growth in several soils when an initially colonized agar food base remained present or was removed. It was concluded that a food base is needed only to initiate but not to maintain saprophytic growth of R. solani (3,33). Growth of R. solani was shown to decrease but still occur as soil moisture levels increased from 33 to 80%. A reduction in soil aeration was one explanation given for this reduced growth of R. solani (3). Saprophytic growth and colonization of organic matter by R. solani were found to be influenced by pH (3,32). Both growth of, and colonization by R. solani occurred over a pH range of 4.5-8.7 with optimal growth taking place between pH 7.0-7.1. Colonization of organic matter declined markedly in acidic soils below pH 4.1.

A number of plant species including alfalfa, barley, buckwheat, corn and oak are saprophytically colonized by R. solani. Mature buckwheat stem pieces were shown to be colonized within four days of being placed into soil (31).

Reduced growth of R. solani in soil amended with organic matter and reduced recovery of R. solani from buried stem pieces initially colonized by R. solani, were both shown to occur over time (3,31). Both these phenomena were explained by the finding of an increased number of other microorganisms, some of which were stated to be possibly antagonistic to R. solani (3,4,31).

ii) Rhizoctonia solani as a plant pathogen

Infection of plants by R. solani is related to the presence of a food base (4). Roots of peanut plants remained healthy when plants were grown in R. solani-infested soil containing very little organic matter. Peanut plants were diseased and had discolored roots when plants were grown in R. solani-infested soil containing peanut roots of a previous crop. It was stated that R. solani utilized the colonized dead roots as food and/or energy sources from which it then infected peanuts. Several factors were said to be

important in determining whether a particular kind of organic matter acted as a food base for R. solani and later disease of peanuts (4): 1.) kind of organic matter, with disease incidence being greatest in soil amended with soybean, then cotton, and least in soil amended with corn plant matter; 2.) depth of organic matter in the soil, with disease incidence decreasing as the organic matter was placed at lower depths in the soil; and 3.) length of time the organic matter is in the soil before peanuts were planted, with disease incidence decreasing as length of time increased between amending soil with organic matter and planting peanut seed. Increasing numbers of microorganisms some of which might be possibly antagonistic to R. solani, and resulting decomposition of the organic matter were two explanations given for this reduction of disease incidence (4).

The pathogenicity of R. solani towards radish in soils containing saprophytically colonized stem pieces was shown to be affected by whether the soils were initially autoclaved or not (32). Rhizoctonia solani had greater effects on reducing seedling emergence and presence of disease symptoms on emerged seedlings in autoclaved soil than in non-autoclaved soil. Another finding was that saprophytic colonization of stem pieces by R. solani in soils previously planted to radish, was greater in initially autoclaved soil than in non-autoclaved soil (32).

Decreased pathogenicity and saprophytic colonization by R. solani in non-autoclaved soil is possibly related to the presence and increase in numbers of other microorganisms in non-autoclaved soil. Some of these microorganisms might have antagonistic effects on R. solani reducing both its pathogenicity and saprophytic ability.

E. Biological control of seedling diseases

In this thesis, biological control refers to the total or partial destruction of pathogen populations by other microorganisms (1). Inherent in this definition is the biological management and reduction of disease incidence. Many reports exist of the successful use of biological control techniques for controlling various seedling diseases.

Studies have shown the effectiveness of non-pathogenic species in controlling pathogenic species of the same genus. Pythium oligandrum Drechsler, which is non-pathogenic, has been shown to control damping-off of sugar beet and cress, both caused by P. ultimum Trow (27,29). Several possible mechanisms reported as being involved in the control of P. ultimum and other Pythium spp., by P. oligandrum include competition for space and nutrients by P. oligandrum applied to seed, alterations in seed exudates resulting from metabolism of P. oligandrum applied to seed, and hyphal interactions involving hyphae of P. oligandrum coiling around and penetrating hyphae of P. ultimum (29).

A non-pathogenic isolate of R. solani was shown to reduce damping-off of wheat caused by a pathogenic R. solani isolate and damping-off of cotton, radish and wheat caused by pathogenic R. solani and R. zeae Voorhees isolates (21). One proposed mechanism for disease control by the non-pathogenic R. solani isolate, was induced resistance or cross-protection in the plants resulting from elicitation of phytoalexins, suberization, or lignification (21).

A number of studies have reported the effectiveness of Penicillium spp. in controlling pathogens of a number of crops. Seedling blight and particularly pre-emergence damping-off of pea caused by a number of fungi including Aphanomyces euteiches Drechsler, Fusarium oxysporum Schlecht, F. solani (Mart.) App. and Wr., Pythium spp., and R. solani, were significantly reduced by applying P. oxalicum Currie and Thom to seed (39). Pre-emergence damping-off was controlled when seeds were coated with $3\text{-}5\text{x}10^6$ spores per seed, but not when seeds were coated with $0.5\text{x}10^6$ spores per seed. The authors proposed that the numbers of viable spores applied to seed, and also the spore germinability in soil, are two factors affecting the control of damping-off of pea by P. oxalicum (39).

Much information exists concerning the abilities of Trichoderma spp. to control seedling diseases of a number of crops.

F. Trichoderma spp. as biological control agents for seedling diseases

Trichoderma spp. are ubiquitous, saprophytic soil fungi. This genus utilizes a wide range of carbon and nitrogen compounds, and sporulates readily on many natural and artificial substrates (30). These abilities aid this fungus in its colonization of substrates, and survival.

A number of studies have employed the use of Trichoderma spp. as seed coatings for the control of soil-borne pathogens. A T. hamatum (Bonord.) Bain isolate, later stated to be T. harzianum Rifai (35), when applied to seed as a spore suspension was found to reduce damping-off of pea and radish, in soils naturally infested with Pythium spp. and artificially infested with R. solani, respectively (19). Incubation of coated radish seed in a moist chamber revealed that the T. hamatum spores had germinated to form hyphal networks and had colonized the seed coats.

Both T. harzianum and T. koningii Oudem. applied as conidia coatings to pea seed reduced the amount of pre-emergence damping-off in a Pythium sp.-infested soil (26). The rate of germination of Trichoderma spp. conidia differed between the two species, and was dependant on temperature. Both Trichoderma spp. isolates underwent abundant sporulation on the seed coats in areas of hyphal interactions with the Pythium sp. The production of toxic metabolites by the Trichoderma spp., was proposed as the mechanism involved in control of Pythium sp. caused damping-off of pea (26). Filtrates from the liquid media in which the Trichoderma spp. were grown, resulted in a decreased growth rate and greatly decreased amount of germination of sporangia of the Pythium sp.

Control of R. solani-caused damping-off of cotton was provided by T. hamatum and T. harzianum applied as spore suspensions to seed (14). Trichoderma hamatum reduced damping-off to a greater degree than T. harzianum, at 20°C. Trichoderma harzianum reduced damping-off to a greater degree than T. hamatum at 27°C. The authors stated that choosing a particular species adapted to specific environmental conditions including

temperature, will affect the ability of that species in controlling a particular disease (14). Further studies could be undertaken to study the application of both species to seed, to provide control at a wider range of environmental conditions. These studies should include experiments to determine whether any detrimental interactions occur between the different isolates/species applied to seed. One possible reason for the difference in degree of control of damping-off of cotton by T. hamatum and T. harzianum at different temperatures (14), is a difference in rate of conidia germination between these two species (26).

Low levels of available iron in soil, and fluorescent pseudomonad bacteria were shown to affect the ability of Trichoderma spp. in controlling damping-off and seed rot of peas (18,20). Of a number of tested Trichoderma spp. coated onto seed, only T. koningii and T. harzianum obtained from a Pythium-infested soil reduced the amount of damping-off and seed rot of pea in this Pythium spp.-infested soil (18). These isolates were adapted to the low levels of available iron in this soil. They were shown to have germinated, colonized, and sporulated on seed coats of peas in this soil (18). Trichoderma hamatum applied as a seed coating controlled pre-emergence damping-off and seed rot of pea caused by Pythium spp. in soil containing 8 µg available iron per g soil and fluorescent pseudomonad bacteria, but not in soils containing less than 4 µg available iron per g soil and these bacteria (20). Fluorescent pseudomonad bacteria by their production of siderophores were shown to reduce the level of available iron in soil. These bacteria adversely affected the growth of T. hamatum and its potential for controlling pre-emergence damping-off and seed rot of pea caused by Pythium spp. Conidia of T. hamatum germinated and the hyphae colonized pea seed coats in soil lacking these bacteria, however the hyphae were frequently lysed and were absent in regions on seed coats where these bacteria were present (20).

Incorporation of Trichoderma sp.-colonized substrates including wheat bran into pathogen-infested soil, has been shown to result in control of specific pathogens and

diseases. Wheat bran/peat mixtures colonized by T. harzianum or T. hamatum resulted in control of damping-off of cucumber, pea and tomato all caused by P. aphanidermatum (Edson) Fitzp. (36). Growth, sporulation, and conidial germination of the Trichoderma spp., were shown to be affected by pH of the growth substrate. Addition of peat to the wheat bran food substrate resulted in an optimal pH of 5.0 for the Trichoderma spp. (36).

Incorporation of a T. harzianum-wheat bran preparation into R. solani-infested soil resulted in control of damping-off of eggplant, tomato and beans (17). Trichoderma harzianum when grown in liquid culture, was found to produce extracellular ß-1,3-glucanase and chitinase which degraded laminarin and chitin (chitin is found in cell walls of R. solani), respectively, and also cell walls of R. solani. Microscopic observations showed degradation of R. solani hyphae by the hyphae of T. harzianum (17).

It has been known since the early 1960's that chitin added to soil can control diseases caused by certain plant pathogens. Amending soil with chitin resulted in significant reductions in both root rot of bean and vascular wilt of radishes caused by F. solani and F. oxysporum, respectively, but no control of damping-off of a number of crops, and crown gall of tomato, caused by P. aphanidermatum (Edson) Fitzp. and Agrobacterium tumefaciens (Sm. and Towns.) Conn, respectively (28). Chitin is known to be present in the cell walls of both Fusarium spp., but not in the Pythium sp., or the Agrobacterium sp. tested. Amending soil with chitin resulted in stimulation of lytic- and antibiotic-producing microbes that were deleterious to the Fusarium spp. (28).

Recent research showed that T. harzianum and T. viride Pers. ex Fr. produce chitinase, and ß-1,3-glucanase (13,34). These enzymes were shown to degrade chitin and ß-1,3-glucan, respectively, both of which are found in cell walls of R. solani and a number of other fungi. Trichoderma harzianum and T. viride produced chitinase and ß-1,3-glucanase when grown in a liquid medium containing chitin or cell walls of R. solani (13,34) and when grown in soil containing R. solani hyphae (13). Various isolates of T.

harzianum and T. viride produced different amounts of ß-1,3-glucanase and chitinase (34). Trichoderma harzianum isolates able to parasitize R. solani produced higher levels of chitinase than an isolate not able to parasitize this pathogen (13). Similarities existed in the composition of proteins produced by five isolates of T. harzianum in response to R. solani cell walls (34). Differences existed between the composition of proteins produced by T. harzianum and T. viride in response to R. solani cell walls. The same isolate of T. harzianum produced different proteins when challenged with different AG's of R. solani (34). Differences amongst isolates of one Trichoderma sp. and amongst different Trichoderma spp., regarding potential to control specific plant pathogens, can therefore be partly attributed to these differences in proteins produced by each in response to specific plant pathogens.

Incorporation of Trichoderma sp. propagules into alginate pellets is a recently developed method for the application of Trichoderma spp. to control plant pathogens (15,24,25). Three important aspects in the production of these pellets are: 1.) incorporation of a food base such as wheat bran to increase viability of potential propagules; 2.) incorporation of chlamydospores rather than conidia to result in greater numbers of viable propagules (24); and 3.) storage of pellets at low temperatures (5°C) to increase viability of the propagules.

Three Trichoderma spp., T. hamatum, T. harzianum and T. viride, incorporated into alginate-bran pellets, were studied for their effects on survival of R. solani, saprophytic colonization of seed by R. solani in soil and control of R. solani-caused damping-off of cotton and sugar beet in soil (25). Amending soils with these three Trichoderma spp. resulted in a significant reduction in survival of R. solani based on regrowth of R. solani from beet seed initially colonized by R. solani, and a significant reduction in colonization of beet seed by R. solani. Damping-off of cotton and sugar beet, was significantly reduced by amending soil with pellets containing T. hamatum and T.

viride (25). These alginate-Trichoderma spp. pellets were found to be effective in controlling R. solani-caused damping-off of cotton and sugar beet.

G. Mechanisms involved in biological control by Trichoderma spp.

Specific hyphal interactions are one type of mechanism involved in antagonism of Trichoderma spp. towards other fungi. A number of Trichoderma spp. including T. hamatum, T. harzianum, T. koningii, and T. viride produced hyphae that coiled around hyphae of F. oxysporum, Fomes annosus (Fr.) Karst., P. ultimum and R. solani. Trichoderma viride hyphae also penetrated, and grew intracellularly inside P. ultimum hyphae (10). Both T. hamatum and T. harzianum underwent hyphal coiling and production of appressorium-like structures on hyphae of R. solani and Sclerotium rolfsii Sacc. Hyphae of S. rolfsii were penetrated by T. hamatum hyphae (12).

Production of cell wall degrading enzymes by Trichoderma spp., is involved in penetration of hyphae of other fungi. Production of cellulase by T. harzianum allows for penetration of Oomycete hyphae containing cellulose, as seen by penetration of hyphae of P. ultimum by T. harzianum (10,13). Chitinase and ß-1,3-glucanase, are two additional extracellular enzymes produced by T. harzianum and T. viride (12,34). These two enzymes were produced when T. harzianum was grown on chitin or cell walls of R. solani and S. rolfsii (13). The ability of T. harzianum to penetrate S. rolfsii hyphae containing chitin and ß-1,3-glucan, is associated with production of increased levels of these two enzymes (12).

Production of non-volatile antibiotics by Trichoderma spp., is a second mechanism involved in their antagonism towards other fungi. Trichodermin is one antibiotic produced by T. polysporum (Link ex Pers.) Rifai and T. viride (8). Trichoderma viride produces a number of other antibiotics including alamethicine, dermadine and suzukacillin. All these antibiotics are both antibacterial and antifungal. Two effects of

these antibiotics on R. solani growing on agar plates were inhibition of colony growth and stimulation of hyphal branching and vacuolation (8).

Volatile metabolite production by Trichoderma spp. is a third mechanism involved in growth inhibition of other fungi. Volatile metabolites produced by T. koningii and T. viride inhibited growth of R. solani, P. ultimum and F. annosus (9). The inhibited fungi were reported to have stunted mycelial growth consisting of more highly branched hyphae than normal. Acetaldehyde, one component of the volatile metabolites produced by T. viride, was shown to inhibit growth of F. annosus indicating that production of this compound by T. viride, can result in growth inhibition of other fungi.

H. Objectives of this Thesis

The objectives of this thesis were: 1.) to identity the fungal pathogens causing CSB in Alberta, 2.) to study the involvement of Pythium spp. especially in the pre-emergence phase of CSB, and 3.) to determine whether CSB caused by R. solani can be controlled by Trichoderma spp.

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II. Identification of pathogens causing canola seedling blight in Alberta*

A. Introduction

Canola/rapeseed (Brassica campestris L., Polish rapeseed, and B. napus L., Argentine rapeseed) is an important crop grown in western Canada. In 1986 it was planted on some 2.8 million hectares in western Canada. Canola seedling blight (CSB) (which includes pre- and post-emergence damping-off) (figure II-1), foot rot, and root rot are some important diseases of seedling and adult canola/rapeseed (canola) plants in this area (31).

In western Canada, Rhizoctonia solani has been reported to be the major pathogen causing damping-off and root rot of canola (21). In addition to R. solani, P. ultimum, Fusarium spp. and Pseudomonas sp. have been isolated from diseased canola plants (1,5,7). Complexes involving several fungal pathogens have been reported to be associated with a number of crop diseases. Fusarium spp., Pythium spp. and R. solani have been reported to cause damping-off, and root rot of alfalfa (20), bean (19), soybean (6), wheat (30), and a number of other plant types.

Canola seedling blight and root rot have been reported to be especially serious and widespread in the Peace River region of Alberta (21). Little information on the CSB disease in Western Canada was available until 1984 when this study was initiated. Since then, several papers on CSB mainly from Saskatchewan and the Peace River region of Alberta, have been published (1,3,4,7,21,22,31).

* includes some information which has been published as: Tewari, J.P., A.I. Calman, and H. Furuya. 1987. Pathogens of the seedling blight of canola in Alberta. in Proceedings of the 7th International Rapeseed Congress, Poznan, Poland, pp. 1248-1253.

The objective of these studies was to identify the fungal pathogens causing CSB in Alberta. Diseased seedlings with CSB symptoms were collected from throughout Alberta. Fungi were isolated from these seedlings, identified and were tested for pathogenicity in causing this disease.

B. Materials and Methods

Collection of Diseased seedlings from the field

In May and June of 1984, canola seedlings (both B. campestris and B. napus) with CSB symptoms (figure II-2) were collected from 73 fields from throughout Alberta. The collected seedlings had hypocotyls with constrictions and/or brown discolored lesions. Field locations ranged from southern Alberta around Lethbridge and Vulcan; central Alberta around Edmonton and Vegreville; and northern Alberta around Fairview and High Level (figure II-3). Within each field, 40 to 50 seedlings were collected along a V-shaped transect line. This transect was no closer than 10 m from the field edge to reduce boundary effects. The collected seedlings having up to the first set of true leaves, belonged to growth stage 1 (8). Seedlings from each field were placed into a polyethylene bag, transported on ice to the laboratory and kept at 4°C for up to several days until isolations were made from them. A total of 2649 canola seedlings with post-emergence symptoms were collected.

Isolation of presumptive pathogenic fungi from seedlings

As many as 40 collected seedlings from each field were washed under running tap water to remove soil debris. All isolation procedures were done aseptically. The seedlings were surface disinfected in 1 percent (%) sodium hypochlorite for one minute, followed by four serial rinses in sterile distilled water. From each seedling the hypocotyl region having the symptoms of CSB was cut into four pieces (each piece being several mm long), with each piece being placed onto a separate petri plate containing one of the

following four agar media: (i) Nash-Snyder (ns), selective for Fusarium spp. (15); (ii) corn meal amended with pimaricin, vancomycin hydrochloride, and pentachloronitrobenzene (cpvp), selective for Pythium spp. (9); (iii) KO (ko), selective for Rhizoctonia spp. (13); and (iv) acidified potato dextrose plus rose bengal (apdarb), a general growth medium (14). Each petri plate contained pieces from three or four seedlings. Petri plates with tissues on ns, ko and apdarb were incubated at room temperature in the light (rtl) or 18-20°C in the dark for cpvp. Obtaining pure cultures of fungal isolates from seedling pieces involved making subcultures on potato dextrose agar (pda), acidified pda (apda) or water agar (wa) until free from bacteria and containing uniform fungal growth. Pure cultures were stored at 4°C on slants of pda or 2.8% V8® juice in 1% agar (v/w)(v8), for Pythium spp. (Dr. D.J.S. Barr, personal communication, 1987).

Identification of fungi from seedlings

Identification of fungi from seedlings isolated on the various four media was carried out to genus level based on colony, hyphal and reproductive characteristics. Representative isolates of Fusarium spp., Pythium spp. and Rhizoctonia spp. were sent to the Biosystematics Research Centre (brc) of Agriculture Canada in Ottawa, and the C.A.B. International Mycological Institute (cmi) in Kew, England to have their identifications confirmed. Many of these same isolates were also tested for pathogenicity to canola.

Pathogenicity testing of fungi from seedlings

i) Canola cvs. used as test plants

Brassica campestris cv. Candle seed was used in all experiments. This seed was first surface disinfected by soaking in 70% ethanol:5% sodium hypochlorite (1:1,v/v) for 30 seconds followed by a series of three rinses in sterile tap water (24).

ii) Selection of fungal isolates

A number of isolates of Fusarium spp., Pythium spp. and R. solani were tested for pathogenicity in causing CSB. Fungi tested: 1.) were from geographically distinct fields, 2.) had differences in morphological appearances of colonies, and 3.) had differences in microscopic appearances of reproductive propagules.

iii) Inoculum production

Inoculum production for all fungi involved aseptic methods. Fusarium spp. isolates were grown on a mixture of oat and wheat seed (2:1, w/w) (23) and R. solani isolates were grown on rye seed (1). Both oat/wheat and rye seed were placed into 1 liter flasks, thoroughly moistened with tap water, and then sterilized twice. The various Fusarium spp. and R. solani isolates were grown on pda petri plates at rtl for five days and then each of the flasks of seed was inoculated with one entire plate of the various Fusarium spp. and R. solani isolates, respectively. All Fusarium spp. and R. solani isolates were grown on seed at rtl for 14 days. The colonized seeds were then air-dried for five days at rtl. The dried seeds were homogenized and then passed through a 0.5 mm pore sieve. The sieved seed inoculum of all isolates was stored at 4°C until used.

Ten cm diameter glass petri plates each containing distilled water:city pond water (2:1, v/v), and two, 2 cm long pieces of corn leaves were sterilized twice, for Pythium spp. inoculum production (29). Pythium spp. isolates were first grown on pda plates at rtl for four days. Colony margins were transferred from isolates growing on pda to petri plates containing the sterilized corn leaves and water. Isolates were incubated in these petri plates for seven days at rtl and then inoculum was collected. For each isolate, the contents from three petri plates were brought to a total volume of 100 ml with sterile distilled water. This solution was homogenized, passed through a 150 µm pore sieve and the filtrate was centrifuged at 5,000 g for 10 minutes at 4°C. The resulting pellet was stored at 4°C until used (10).

iv) Numbers of viable propagules in inocula of Fusarium spp., R. solani and Pythium spp.

Numbers of viable propagules referred to as colony forming units (cfu), were determined for the dried inoculum of the Fusarium spp. and R. solani isolates. A 0.1 g portion of inoculum of each isolate was diluted in a series of sterile water blanks. Dilutions were plated onto three apda petri plates. The plates were incubated at rtl for four days, by which time colonies (cfu) had developed and were counted.

Viable propagule numbers (cfu) were obtained for inocula of Pythium spp. isolates. The inoculum pellet of each isolate was diluted in a series of sterile water blanks. One ml aliquots of the 1/10,000 dilution of each isolate were evenly spread over each of three pda plates. The plates were incubated at rtl for four days, by which time colonies had developed and cfu were counted.

v) Determination of inoculum levels for pathogenicity experiments

Experiments were undertaken to determine the inocula levels of Fusarium spp., Pythium spp. and R. solani resulting in 50% emergence of canola seedlings. These levels were then used in all subsequent pathogenicity tests of the various isolates. Equal mixtures of inocula of two Fusarium spp. isolates K1.33 and N2.9A, or two R. solani isolates K2.13 and K2.15, were used in one experiment. Various amounts, 0.1 g, 0.3 g, 1 g, 2 g, 5 g and 8 g of the combined inoculum were mixed with 5 kg of previously sterilized greenhouse soil mix (soil) (1:2:1, soil:peat:sand, v/v/v). Sterilized soil was placed into 9 cm x 9 cm peat pots and pressed firm to fill them 3/4 full. Ten seeds were evenly distributed over the soil. Seeds in each pot were then covered to a depth of 2.5 cm with either sterilized soil (controls) or with soil infested with Fusarium spp. or R. solani. Each of the treatments; controls, soil infested with each of the six levels of Fusarium spp. and soil infested with each of the six levels of R. solani was replicated in

eight pots. Soil in all pots was watered with tap water to saturation. The experiment was set up as a completely randomized design, in a growth cabinet adjusted to 18°C day/7°C night, with a 16 hour photoperiod. Soil in the pots was watered with tap water when needed. Seedling emergence and number of seedlings with CSB symptoms were recorded eight days after planting. Symptoms included hypocotyls that were tapered, constricted and/or with brown discoloration.

For determination of Pythium spp. inoculum level, a mixture of inoculum pellets of five Pythium spp. isolates diluted from 1/250-1/250,000 was applied onto soil at a distance of 1 cm from planted seed and covered with an additional 1.5 cm of soil. Resulting emergence was less than 23% for any of the pellet dilutions. An additional experiment studied the effects of 1.) two levels of inoculum, and 2.) placement of inoculum at two distances from the planted seed. Two Pythium spp. isolates C1.3.8 and P66.2.2, were chosen for this experiment. Methods for production and collection of oospores and sporangia were as previously described. These two isolates were individually tested. The pellet of each isolate was diluted with sterile distilled water to 1/200 and 1/1000 dilutions. Filling of pots with soil, number of seed per pot and establishment of control pots were as previously described. For pots containing soil amended with either of the two isolates, the inoculum was added in either of two manners: 1.) seed was covered with soil to a depth of less than 0.5 cm, then a 25 ml aliquot of the 1/1000 dilution of that isolate was evenly distributed over the soil, followed by the addition of enough soil to cover the seed to a final depth of 2.5 cm, and 2.) seed was covered with soil to a depth of 2.5 cm, followed by a 25 ml aliquot of the 1/200 dilution of that isolate being evenly distributed over the soil. The controls and each of the two inoculum treatments described above were replicated in four pots. The experiment was set up as a completely randomized design in one growth cabinet. The conditions in this cabinet, watering of pots, length of experiment and data collected were the same as those previously reported.

vi) Testing selected isolates of Fusarium spp., Pythium spp. and R. solani for pathogenicity to cv. Candle

A number of isolates of Fusarium spp., Pythium spp. and R. solani were each tested for pathogenicity to Candle. Filling of pots with soil, number of seed per pot and establishment of control pots were as previously described. Soil was infested with each Fusarium sp. isolate at a rate of 20 g inoculum per 10 kg soil; with each R. solani isolate at a rate of 2 g inoculum per 10 kg soil; and with each Pythium sp. isolate by drenching a 0.5 cm deep layer of soil above the seed with 25 ml of a 1/1000 dilution of an inoculum pellet of that isolate. Seeds in all pots were covered to a final soil depth of 2.5 cm. Each of the treatments was replicated in eight pots. The experimental design was a completely randomized design in one growth cabinet, with cabinet conditions, watering of pots and type of data collected being the same as those previously reported. Data was collected 11 to 14 days after planting. All data of % emergence and % seedlings with CSB symptoms were subjected to arcsin transformations prior to ANOVA, with means being compared by LSD. Seedlings with CSB symptoms from soil infested with various isolates of Fusarium spp., Pythium spp. or R. solani were aseptically plated onto ns, pda or ko, respectively, for reisolation of fungi. Individual dot lesions on cotyledons of seedlings from Pythium-infested soil and cotyledons without these lesions from seedlings from non-infested soil were aseptically plated onto pda. All seedling tissues were surface disinfected in 0.5% sodium hypochlorite for 30 seconds, rinsed in three serial washes of sterile distilled water and then plated onto the various media. Tissues were incubated at rtl until microscopic examinations were made of the developing fungal colonies.

C. Results

Isolation of presumptive pathogenic fungi from seedlings

Table II-1 shows data for the numbers of canola seedlings plated on all four agar media and numbers of fungi found colonizing these seedlings. Rhizoctonia spp., Fusarium spp. and Pythium spp. isolates (total was 1315) comprised 57.2% of the total number of isolated fungi (2299). Rhizoctonia spp. isolates were the most numerous of these three fungi, comprising 33.8% (777); Fusarium spp. comprised 17.6% (404); and Pythium spp. was least numerous, comprising 5.8% (134) of the total number of fungi. The remaining 984 fungal colonies (42.8% of total fungi), among other fungi included species of Aspergillus, Cephalosporium, Humicola, Mucor, Penicillium, Rhizopus and Trichoderma.

Identification of fungi from seedlings

The following fungi were isolated from canola seedlings having CSB symptoms:

1.) Fusarium acuminatum Ellis and Everhart

2.) F. avenaceum (Fries) Saccardo

3.) Pythium paroecandrum Drechsler

4.) P. sylvaticum Campbell and Hendrix

5.) Rhizoctonia solani Kuhn including AG 2-1, and AG 4

Fusarium acuminatum K51.4. Colonies on pda pinkish-yellow-white and containing thick aerial mycelium; colony reverse dark purple-red-brown. Macroconidia produced in large numbers, measuring 27-33 x 4-5 µm, containing 1-2 septa, and having curved terminal cells. Microconidia fewer in number, measuring 10-15 x 3-4 µm, and 1-celled. Chlamydospores present in hyphae and macroconidia.

Fusarium avenaceum P66.30. Colonies on pda orange-brown with some red-purple coloration; aerial hyphae absent. Macroconidia produced in large numbers, measuring 39-69 x 3-4 µm, containing 3-5 septa, curved towards terminal cells and

one terminal cell hooked with narrow tip. Microconidia fewer in number, measuring 21-24 x 4 µm and 1-celled. Chlamydospores present in macroconidia.

Pythium paroecandrum C1.3.8. Colonies on corn meal agar (cma), producing white aerial mycelium. Sporangia intercalary or terminal, globose and 24-27 µm diameter. Oogonia mostly intercalary, globose, smooth-walled, 18-23 µm diameter and each containing one aplerotic globose oospore of 15-20 µm diameter.

Pythium sylvaticum C32.12. A heterothallic species. Colonies on cma producing white cottony aerial mycelium. Hyphal swellings intercalary or terminal, globose or limoniform and being 21-27 µm in diameter or 15 x 24 µm in size, respectively. No oogonia or antheridia seen in single cultures.

Rhizoctonia solani. Colonies on pda brown-black, with brown-black sclerotia. Hyphae 9-11 µm in width, branching at 90° relative to main hyphae. Hyphal branches each with a constriction and septum at the base. R. solani isolates included AG 2-1 and AG 4 (22).

Pathogenicity testing of fungi from seedlings

iv) Numbers of viable propagules in inocula of Fusarium spp., R. solani and Pythium spp.

The dried inoculum of the tested Fusarium spp. and R. solani isolates, contained 0.9 x 10^7 to 140 x 10^7 cfu per gram, and 1.5 x 10^4 to 38.5 x 10^4 cfu per gram, respectively. Pellets of inocula of three Pythium spp. isolates contained 1.9 x 10^5 to 3.0 x 10^5 cfu/ml.

v) Determination of inoculum levels for pathogenicity experiments

The following results were obtained from studies to determine the optimal inoculum levels for the various Fusarium spp., Pythium spp. and R. solani isolates:

a.) Fusarium spp. Candle emergence was 82.5% in non-infested soil. Increasing levels of Fusarium spp. inoculum (inoculum containing 7.4 x 10^6 cfu/g) resulted in decreased emergence of Candle. The highest level of inoculum 8 g/5 kg soil; resulted in 71.3% emergence. None of the seedlings had CSB symptoms, for any of the tested inoculum levels. Based on these results all experiments involved addition of 10 g inoculum/5 kg soil for each Fusarium sp. isolate.

b.) Rhizoctonia solani. Candle emergence was 91.3% in non-infested soil. Increasing levels of R. solani inoculum (inoculum containing 2.4 x 10^4 cfu/g) resulted in decreased emergence of Candle. Infesting soil with 1 g inoculum/5 kg soil resulted in 53% emergence and 55.9% of the seedlings having CSB symptoms. Based on these results all experiments involved addition of 1 g inoculum/5 kg soil for each R. solani isolate.

c.) Pythium spp. Candle emergence was 75% in non-infested soil.The 1/1000 dilution of inoculum of Pythium spp. isolates C1.3.8 and P66.2.2 when placed at a distance of 0.5 cm from the Candle seed, resulted in 52.5% and 5% emergence, respectively. Infesting soil in this manner with isolate P66.2.2 resulted in 100% of the seedlings having CSB symptoms. Based on these results, the inoculation procedure for later experiments involved a 1/1000 dilution of Pythium sp. inoculum being applied as a 25 ml drench within 0.5 cm of the planted seed.

vi) Testing selected isolates of Fusarium spp., Pythium spp. and R. solani for pathogenicity to cv. Candle

A total of eight R. solani isolates were tested for pathogenicity in causing CSB of cv. Candle. These R. solani isolates all belonged to AG 2-1 (23). Four isolates from southern Alberta, P4.1, N9.1, N10.27 and P22.8, were individually tested in one group (Table II-2). Seedling emergence was 83.8% in soil not infested with R. solani. These four isolates were pathogenic in causing CSB of Candle. All these isolates resulted in significant reductions in percent emergence which ranged from 3.8 to 20%. Seedlings

from soil not infested with R. solani (control), had no CSB-like symptoms. All four R. solani isolates significantly increased the percent of seedlings having CSB symptoms, to 100%.

Two isolates from central Alberta C51.25 and N69.32, and two isolates from northern Alberta P32.2 and K49.16, were individually tested as one group (Table II-3). Seedling emergence was 96.3% in soil not infested with R. solani. These four isolates were pathogenic in causing CSB of Candle. All isolates resulted in significant reductions in percent emergence, which ranged from 1.3 to 20%. In soil not infested with R. solani, 1.3% of the seedlings had CSB-like symptoms (explanation for this in D. Discussion). All four R. solani isolates significantly increased the percent of seedlings having CSB symptoms, to levels of 96.9 to 100%. All eight R. solani isolates were re-isolated from seedlings with CSB symptoms. Typical R. solani hyphae had developed from the plated tissues of seedlings exposed to all of the eight R. solani isolates.

A total of 10 Fusarium spp. isolates were tested for pathogenicity in causing CSB of the cv. Candle. Table II-4 shows results from the pathogenicity testing of five Fusarium spp. isolates K6.27, N15.6, K51.4, N59.11 and P66.30, from central Alberta. In non-infested soil (control), seedling emergence and seedlings with CSB-like symptoms were 85.0% and 9.8%, respectively. Two isolates, K6.27 and P66.30 resulted in significant reductions in percent emergence of Candle. Isolate P66.30 identified as F. avenaceum, lowered percent emergence to 65% and also resulted in a significant increase in seedlings with CSB symptoms (43.2%) compared to the control treatment. Isolate K6.27 lowered percent emergence to 63.8%. Isolate N15.6, also identified as F. avenaceum, resulted in a significant increase in seedlings with CSB symptoms (44.1%). Fusarium acuminatum K51.4, was not pathogenic to Candle. It had no significant effect on % emergence (80%) or % seedlings with CSB symptoms (0%) at the inoculum level used (Table II-4).

Table II-5 shows results from the pathogenicity testing of five Fusarium spp. isolates, K33.38, K35.8, N39.33, K49.36 and C71.7 from northern Alberta. In non-infested soil (control), seedling emergence and emerged seedlings with CSB-like symptoms were 92.5% and 0%, respectively. One isolate K33.38 resulted in a significant difference in both % emergence (to 61.3%) and % seedlings with CSB symptoms (to 50.9%) compared to the control treatment (Table II-5).

A total of four Fusarium spp. isolates were found to be pathogenic in causing CSB of the cv. Candle. They resulted in significant changes in % emergence and/or % seedlings with CSB symptoms. Three Fusarium spp. isolates, K6.27, K33.38 and P66.30 resulted in significant reductions in percent emergence of the cv. Candle. The isolates K33.38, P66.30 and also N15.6 all resulted in significant increases in percent seedlings with CSB symptoms. All ten Fusarium spp. isolates were reisolated from seedlings with CSB symptoms.

Table II-6 shows results of the pathogenicity testing of two Pythium spp. isolates C1.3.8 and C32.12, in causing CSB of the cv. Candle. Isolates C1.3.8 and C32.12, identified as P. paroecandrum and P. sylvaticum, respectively, were from southern and northern Alberta, respectively. In non-infested soil (control) seedling emergence was 83.8% and 5.1% of the seedlings had CSB-like symptoms. Both these Pythium spp. isolates were pathogenic in causing CSB of the cv. Candle. Isolate C32.12 resulted in a significant reduction in % emergence (to 52.5%) and a significant increase in % seedlings with CSB symptoms (to 81.8%), and isolate C1.3.8 resulted in a significant increase in % seedlings with CSB symptoms (to 80.2%) compared to the control (Table II-6).

One distinctive symptom on emerged seedlings from Pythium-infested soil, was the presence of brown dot lesions on the cotyledons. Both Pythium spp. were reisolated from cotyledon pieces having dot lesions and from seedling hypocotyls having lesions and

necrosis. No fungus grew from seedling pieces taken from seedlings grown in non-infested soil.

D. Discussion

Canola seedling blight (CSB) was found to be a widespread disease in Alberta, based on field sampling done in 1984. Seedlings exhibiting CSB symptoms were found in all the 73 commercial fields sampled throughout Alberta. Incidence of CSB varied amongst and within these fields.

Rhizoctonia spp., Fusarium spp., and Pythium spp. were isolated from canola seedlings showing CSB symptoms, from 71 of the 73 fields. Of these three genera, Rhizoctonia spp. were the most frequently isolated, followed by Fusarium spp., and then Pythium spp. (33.8%, 17.6%, and 5.8% of total fungi, respectively) (Table II-1).

Recent results in Saskatchewan on a study of post-emergence damping-off and root rot of canola seedlings, indicated that Rhizoctonia spp., Fusarium spp. and Pythium spp. represented 31%, 21% and 10% of the total number of fungal isolates (31). These results are similar to the results obtained from Alberta for isolation of fungi from collected canola seedlings exhibiting CSB symptoms.

In these studies, each selective medium was not completely selective for only one fungus genus. Several fungal genera were able to grow on each medium: Fusarium spp. was isolated on cpvp (selective for Pythium spp.) and on ko (selective for Rhizoctonia spp.) in addition to the Fusarium-selective ns medium. Rhizoctonia solani was isolated on ns and on cpvp in addition to the Rhizoctonia-selective medium.

In this study, the fungi isolated from canola seedlings with CSB symptoms were: F. acuminatum, F. avenaceum, P. paroecandrum, P. sylvaticum and R. solani. Past studies from the prairie provinces in Canada, have reported R. solani and a number of Fusarium spp., Pythium spp. and other fungi as being associated with root and stem rot and damping-off of canola. Rhizoctonia solani was reported to be a major canola pathogen

causing damping-off and root rot (21). Fusarium acuminatum, F. equiseti (Cda.) Sacc. and F. solani (Mart.) App. and Wr. were isolated from rapeseed with root and stem rot. These were all pathogenic to rapeseed seedlings (28). These three Fusarium spp. along with F. poae (Pk.) Wr. were reported to be associated with stem rot and foot rot of rapeseed (18). Fusarium oxysporum Schlecht, F. roseum Lk. emend. Snyd. and Hans., F. roseum Avenaceum, F. roseum Culmorum and F. roseum Gibbosum were all isolated from roots of diseased rapeseed. All were pathogenic, except F. roseum Culmorum which was non-pathogenic in causing seedling death (2). In a study of root rot, F. acuminatum, F. oxysporum, F. reticulatum Mont. and F. tricinctum Cda. were isolated from canola seedlings and P. ultimum was isolated from canola stubble (7). A number of the Fusarium spp. isolates did not cause pre- or post-emergence damping-off of B. campestris cv. Tobin or B. napus cv. Westar. Pythium ultimum had no effect on emergence but resulted in disease symptoms including discoloration, on cvs. Tobin and Westar (7). Stem rot of rapeseed has been reported to be caused by Pythium debaryanum Hesse (26). Two other fungi, Pellicularia praticola (Kotila) Flentje and Rhizopus stolonifer (Ehrenb. ex Fr.) Lind were found to cause damping-off and pre-emergence rotting, respectively, of rapeseed (17 and 27, respectively).

Pathogenicity experiments of Pythium spp. involvement in seedling blight of cotton and damping-off of chickpea and involved incorporation of inoculum throughout the soil in which the seed was planted (11,12). In testing the pathogenicity of Fusarium spp. and R. solani in causing CSB, the inoculum was applied throughout the soil above the planted seed. The inoculum of Pythium spp. was applied at a distance of 0.5 cm from the planted seed. These methods resulted in the seed and developing seedlings being immediately exposed to the various fungi.

Data for both % emergence and % seedlings having CSB symptoms were collected in the pathogenicity experiments. This data represents both pre- and post-emergence damping-off, respectively. Both pre- and post-emergence damping-off are components

of CSB (22). Some control seedlings had CSB-like symptoms (Tables II-3, II-4, II-6). These seedlings had discolored tapered hypocotyls and non-germinated seed were soft and mushy. This data was collected so that consistency in observations could be maintained. Canola seedling blight-like symptoms occurred on less than 10% of the emerged control seedlings in any of these three experiments. None of these seedlings yielded any Fusarium spp., Pythium spp. or R. solani upon being plated onto media indicating that these seedlings were not infected with any of these fungi. Possible explanations for these seedlings appearing with csb-like symptoms are injury while growing through soil, natural discoloration or tapering of hypocotyls and/or possible seedborne fungi that weren't eliminated during seed surface disinfestation procedures.

All eight R. solani AG 2-1 isolates when infested into soil at a level of 2 g inoculum/10 kg soil, showed extreme virulence in causing CSB of the cv. Candle (Tables II-2 and II-3). They resulted in high levels of pre-emergence (1.3 to 20% emergence) and post-emergence damping-off (96.9 to 100% of seedlings with CSB symptoms). Thus, R. solani AG 2-1 is pathogenic in causing CSB of the cv. Candle. In a study of root rot of canola in the Peace River region of Alberta, R. solani AG 2-1 resulted in 1 to 42% emergence of the cv. Westar (7). These results are similar to the findings in this study in which eight R. solani AG 2-1 isolates resulted in 1.3 to 20% emergence of the cv. Candle.

Four of the 10 tested Fusarium spp. isolates were pathogenic in causing CSB of the cv. Candle (Tables II-4 and II-5). Both % emergence and % seedlings with CSB symptoms were significantly different in soil infested with isolates K33.38 and F. avenaceum P66.30, than in non-infested soil. Isolate K6.27 resulted in a significant reduction in % emergence. A second F. avenaceum isolate N15.6, resulted in a significant increase in % seedlings with CSB symptoms.

Both F. avenaceum N15.6 and P66.30, were pathogenic in causing CSB of the cv. Candle (3). Fusarium avenaceum P66.30 is possibly more virulent than F. avenaceum

N15.6. Both F. avenaceum P66.30 and N15.6 resulted in similar % emergence (65% and 68.8%, respectively) and similar % seedlings with symptoms (43.2% and 44.1%, respectively) (Table II-4), even though P66.30 was infested into soil at a far lower number of viable propagules than N15.6, (3.4×10^6 cfu/100 g soil and 2.8×10^8 cfu/100 g soil, respectively).

Fusarium acuminatum K51.4 was found to be non-pathogenic in causing CSB of the cv. Candle (Table II-4). Infesting soil with K51.4 did not result in a significant difference in % emergence compared to that in non-infested soil. None of the seedlings had CSB symptoms from soil infested with K51.4. Non-pathogenicity of F. acuminatum to cvs. Candle/Westar was shown in the study of root rot of canola in the Peace River region of Alberta (7).

Both P. paroecandrum C1.3.8 and P. sylvaticum C32.12 were found to be pathogenic in causing CSB of the cv. Candle (Table II-6). Pathogenicity of P. paroecandrum and P. sylvaticum was previously reported in a related study (4). Infestation of soil with P. sylvaticum C32.12, resulted in a significant reduction in % emergence and also a significant increase in % of seedlings with CSB symptoms compared to non-infested soil. Soil infestation with P. paroecandrum C1.3.8, resulted in a significant increase in % of seedlings with CSB symptoms. Both Pythium spp. resulted in more than 80% post-emergence damping-off.

Soil was infested with the same amounts of inocula for all Fusarium spp., Pythium spp. and R. solani isolates. These isolates produced differing numbers of viable propagules (cfu) and hence, the various inocula contained differing cfu. Results of the effects of each of the various isolates in causing CSB were compared to results of the controls, with no comparisons being made amongst the isolates. Further pathogenicity studies should involve testing all isolates of each genus at the same inoculum level to allow for comparisons to be made amongst the isolates concerning their relative virulence in causing CSB.

Table II-1. Numbers of fungal isolates from seedlings collected from fields from throughout Alberta

Region	No. fields sampled	No. seedlings[o] plated	Total no. fungi obtained	Nos. fungal colonies isolated[x] R. spp.	E. spp.	P. spp.	Others
south	18	642	461	182	60	23	196
central	33	1127	710	281	71	55	303
north	22	880	1128	314	273	56	485
Totals	73	2649	2299	777	404	134	984

[o] seedlings

[x] R. Rhizoctonia; E. Fusarium; P. Pythium

Table II-2. Pathogenicity determinations for four R. solani isolates from southern Alberta towards cv. Candle

Treatment	% Emergence[+]	% seedlings with CSB symptoms[s]
Control[o]	83.8	0.0
N10.27	20.0 *	100.0 *
N9.1	11.3 *	100.0 *
P22.8	7.5 *	100.0 *
P4.1	3.8 *	100.0 *

inoculum levels, cfu (x 10^4)/g: P4.1, 28.5; N9.1, 38.5; N10.27, 3.5; P22.8, 6.5

[o] non-infested soil

[+] means based on 8 replicate pots

[s] means based on only those replicate pots with emerged seedlings

* significantly different from control, at $P<0.005$, according to LSD test on transformed data

Table II-3. Pathogenicity determinations for four R. solani isolates from central and northern Alberta towards cv. Candle

Treatment	% emergence+	% seedlings with CSB symptoms[S]
Control[O]	96.3	1.3
N69.32	20.0 *	96.9 *
K49.16	7.5 *	100.0 *
P32.3	5.0 *	100.0 *
C51.25	1.3 *	100.0 *

inoculum levels, cfu (x 10^4)/g: P32.3, 8.5; K49.16, 1.5; C51.25, 20.0; N69.32, 10.0

O non-infested soil

+ means based on 8 replicate pots

S means based on only those replicate pots with emerged seedlings

* significantly different from control, at $P<0.005$, according to LSD test on transformed data

Table II-4. Pathogenicity determinations for five Fusarium spp. isolates from central Alberta towards cv. Candle

Treatment[F]	% emergence[+]	% seedlings with CSB symptoms[S]
N59.11	91.3	28.5
Control[O]	85.0	9.8
K51.4	80.0	0.0
N15.6	68.8	44.1 *
P66.30	65.0 *	43.2 *
K6.27	63.8 *	16.1

[F] K51.4, F. acuminatum, N15.6, P66.30, F. avenaceum, inoculum levels, cfu (x 10^7)/g: K6.27, 4.7; N15.6, 140.0; K51.4, 1.0; N59.11, 0.9; P66.30, 1.7

[O] non-infested soil

[+] means based on 8 replicate pots

[S] means based on only those replicate pots with emerged seedlings

* significantly different from control, at $P<0.005$, according to LSD test on transformed data

Table II-5. Pathogenicity determinations for five Fusarium spp. isolates from northern Alberta towards cv. Candle

Treatment	% emergence+	% seedlings with CSB symptoms^{S}
Control^{O}	92.5	0.0
K49.36	91.3	14.3
N39.33	83.8	5.4
C71.7	81.3	11.0
K35.8	78.8	6.3
K33.38	61.3 *	50.9 *

inoculum levels, cfu (x 10^7)/g:
K33.38, 15.0; K35.8, 1.9; N39.33, 121.4; K49.36, 41.7; C71.7, 12.7

O non-infested soil

\+ means based on 8 replicate pots

S means based on only those replicate pots with emerged seedlings

* significantly different from control, at $P<0.05$, according to LSD test on transformed data

Table II-6. Pathogenicity determinations for two Pythium spp. isolates from northern and southern Alberta towards cv. Candle

Treatment^{P}	% emergence+	% seedlings with CSB symptoms^{S}
Control^{O}	83.8	5.1
C1.3.8	76.3	80.2 *
C32.12	52.5 *	81.8 *

P C1.3.8, P. paroecandrum; C32.12, P. sylvaticum
inoculum levels, cfu (x 10^5)/ml: C1.3.8, 2.0; C32.12, 3.0

O non-infested soil

\+ means based on 8 replicate pots

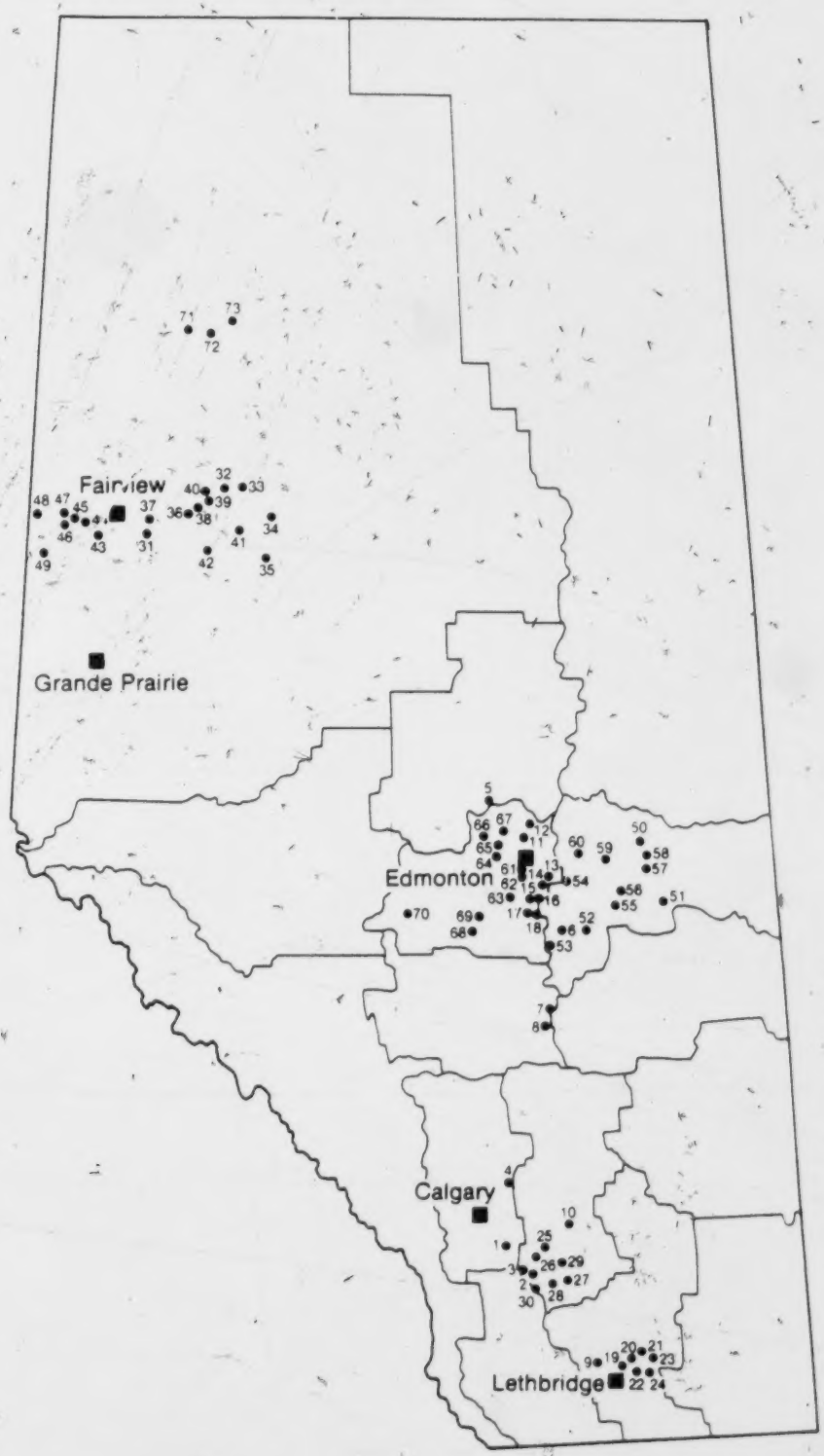
S means based on only those replicate pots with emerged seedlings

* significantly different from control, at $P<0.005$, according to LSD test on transformed data

Figure II-1. (upper). Alberta canola field with non-uniform seedling emergence associated with the seedling blight disease.

Figure II-2. (lower). Canola seedlings with constricted and discolored hypocotyls, two symptoms of the seedling blight disease..

Figure II-3. Map of Alberta showing locations of 73 canola fields from which seedlings having symptoms of the seedling blight disease were collected.


Fairview
Grande Prairie
Edmonton
Calgary
Lethbridge

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III. Involvement of Pythium spp. especially in the pre-emergence phase of canola seedling blight: identification and pathogenicity of Pythium colonizing canola seed

A. Introduction

Pythium spp. are widely distributed throughout the world in both undisturbed and disturbed soils. Pythium spp. subsist in the soil by both parasitic and saprophytic means. As plant pathogens, Pythium spp. are able to parasitize young succulent tissues (11).

A number of Pythium spp. have been isolated from and cause disease of a wide range of crop species (2,8,11,14,19,21). Pythium spp. by themselves, cause diseases including seed decay and root rot of wheat (5), and crown rot of apple (13). Pythium spp. are also involved in disease complexes with other pathogens including Fusarium spp. and R. solani, in causing seedling diseases of a number of crops (6,7,12).

Canola is subject to infection by Pythium spp. Pythium ultimum in addition to R. solani and Fusarium spp., was isolated from canola plants with root rot in Central Alberta (9,18). Pythium polymastum Drechsler, was isolated from and found to be pathogenic to oilseed rape (22). Pythium paroecandrum Drechsler, P. sylvaticum Campbell and Hendrix, and Pythium sp. Group G Van der Plaats-Niterink were isolated from and reported to be involved in the pre-emergence phase of CSB (3).

A small number of Pythium spp. isolates (5.8% of total number of fungi) were obtained from emerged canola seedlings with CSB symptoms collected from fields throughout Alberta in 1984 (3; Chapter II, Table II-1). These seedlings represented the post-emergence damping-off phase of CSB.

Very little information exists regarding the involvement of Pythium spp. in CSB (including the pre-emergence damping-off phase) in Western Canada. Baiting soils with canola seed is one method that could facilitate the isolation of Pythium spp. able to

colonize canola seed/seedlings and cause CSB. Other studies have employed the use of seed of lettuce, onion, chickpea and cucumber as bait in field soils for the isolation of Pythium spp. involved in damping-off (especially the pre-emergence phase of damping-off), and enumeration of Pythium spp. populations (2,16,23).

Studies were undertaken for the purpose of isolation, identification and testing the pathogenicity of Pythium spp. in causing CSB. Isolation of Pythium spp., involved using canola seed as bait in field soils.

B. Materials and Methods

Collection of field soils

Soils from 24 different fields planted to canola in Alberta, were sources of Pythium spp. Soils were collected in May/June, 1984, and June, 1988, from five fields from southern Alberta, around Vulcan; 14 fields from central Alberta, around Edmonton; and five fields from northern Alberta, around Fairview. Within each field, soil was collected by the same procedure as that used for collection of diseased field seedlings (Chapter II, Materials and Methods). All the soil from each field was bulked together and stored in a closed plastic bag at room temperature until used.

Canola cvs. used in experiments

Brassica campestris cv. Candle seed was used as bait in all field soils. Both B. campestris cvs. Candle and Tobin seed were used in the pathogenicity trials. All seed was initially surface disinfected by previously described methods (Chapter II, Materials and Methods).

Isolation of Pythium spp. from field soils

The following methods were employed for all soils collected in 1984 and 1988. Petri plates and mesh screening were initially sterilized. Sterile distilled water was

used throughout. Field soil was placed to a depth of 1 cm in each of four, 13.5 cm diameter glass petri plates. In each petri plate, the soil was thoroughly moistened with water. A single layer of mesh screen having 1 mm^2 pores, was placed on the soil in each petri plate. A number of Candle seed were evenly distributed over the mesh surface and enough water was added to keep the seed in a flooded condition. The petri plates were closed and were placed into one growth cabinet at 7°C night/18°C day, with 16 hours of light.

Seeds were removed from the soils after three days incubation. Enough seed/seedlings (seed) were removed from the petri plates so that 40 or 20 seed were plated from each of the soils from 1984 and 1988, respectively. Soil was removed from the seed by washing them under running tap water for two to three minutes. All the remaining procedures were aseptically performed. The seeds were surface disinfected by soaking in 0.5% sodium hypochlorite for 30 seconds, followed by rinsing in three serial water washes. Seeds from each soil were divided into groups containing five seed per group. All groups of seed were placed into petri plates containing cpvp, which was selective for Pythium spp. and other phycomycetes (13). All plates were incubated at 18°C in the dark for seven to ten days. Data was collected for numbers of colonized seed.

Single hyphal tip cultures of possible Pythium spp. were obtained from colonies developing from seed. Margins from colonies growing on the cpvp plates were transferred to 1.5% (w/v) water agar (wa) plates. Single hyphal tips were taken from morphologically uniform colonies growing on wa, and were transferred to pda plates. Colonies growing on pda were observed for the presence of aseptate hyphae and also uniformity in hyphal diameter. Margins from single hyphal tip pure colonies were then transferred to 2.8% V8® juice in 1.5% agar(v/w) slants and colonies were stored at 4°C.

Identification of Pythium spp. from colonized canola seed bait

Single hyphal Pythium spp. isolates were identified by either of the following two methods: 1.) the use of a taxonomic monograph on the genus Pythium (21), or 2.) sending isolates to the Agriculture Canada Biosystematic Research Centre (brc), or the C.A.B. International Mycological Institute (cmi). Isolates to be identified were chosen based on: a) differences in microscopic appearances of sporangia and oogonia, and b) differences in colony morphologies.

For identification of Pythium spp. by use of the monograph, selected isolates were cultured by a previously described method (Chapter II, Materials and Methods). Isolates produced both sporangia/zoosporangia, antheridia, oogonia and oospores by this culture method. Isolates were also grown on cma. Comparisons were made between colony morphologies of these isolates grown on cma and descriptions of morphologies of known species grown on cma.

Testing pathogenicity of Pythium spp. and other phycomycetes toward canola

A number of phycomycetes including Pythium spp. were isolated from canola seed bait. These fungi were tested for their pathogenicity in causing CSB. Two types of tests were employed: i) a water agar plate method, and ii) a soil method.

i) Water agar (wa) plate method

This procedure involved seed being placed around colony margins of a number of isolates that were individually grown on 1.5% wa plates (4). Each isolate was first grown on pda plates. Several five mm diameter plugs were taken from margins of colonies on pda plates and each plug was transferred to the centre of one of five wa plates. Brassica campestris cv. Tobin seed were surface disinfected and rinsed by previously described methods. In each of the five wa plates containing each isolate, 10 Tobin seed

were placed into the agar, at a distance of 3-5 mm from the colony margin. Control plates involved 10 Tobin seed being placed into each of five non-inoculated wa plates. For each isolate, data was collected for numbers of germinated seed and also numbers of seed/seedlings with CSB symptoms, after four to six days of incubation. Seed germination data was subjected to arcsin transformations prior to ANOVA, with means being compared by the Duncan's multiple range (dmr) test.

ii) soil pathogenicity tests

Selected fungal isolates were cultured and inoculum was collected by a previously described method (Chapter II, Materials and Methods). Colony forming unit determinations were done for each of the isolates. Each isolate was diluted in sterile distilled water and the dilutions were plated onto three or four pda plates. Colonies were counted after the plates were incubated for 24 hours at rtl.

All isolates were tested by the same procedures, except for the following noted exceptions. Isolates from 1984 and 1988 soils were tested against B. campestris cvs. Candle or Tobin, respectively. All seed were surface disinfected by previously described methods. Ten or 25 seeds were evenly distributed over firmed sterilized greenhouse soil mix (1:2:1, soil:peat:sand, v/v/v) (soil) in 10 cm^2 plastic pots or 16 cm^2 peat pots, for the testing of the isolates from the 1984 and 1988 soils, respectively. Filling of pots with soil, establishment of control pots, infesting soil with each Pythium sp. isolate., number of replicate pots per treatment, experimental design, growth cabinet conditions, watering of pots and collection of data were the same as previously described (Chapter II, Materials and Methods, vi). All data were subjected to arcsin transformations prior to ANOVA, with means being compared by LSD.

C. Results

Isolation of Pythium spp. from field soils

In all field soils collected in both years, B. campestris cv. Candle seed had become colonized by fungi able to grow on the Pythium-selective medium. This had resulted within three days after the seed was placed as bait in these soils. In the 19 field soils from 1984, colonies grew from 269 (35.4%) of the 760 canola seed. The numbers (and percentages) of colonized seeds per field ranged from 4 (10%) to 25 (62.5%) of 40 seeds. In the five field soils from 1988, colonies grew from 80 (80%) of the 100 canola seeds. The numbers (and percentages) of colonized seed per field ranged from 14 (70%) to 17 (85%) of 20 seeds. In addition to Pythium spp. colonizing the seed, other fungi also colonized the seed. Rhizoctonia spp. had colonized at least 11% of the seed used as bait in the soils collected in 1988.

Identification of Pythium spp. from colonized canola seed bait

Pythium pythioides (Roze and Cornu) Ramsbottom, P. rostratum Butler, P. ultimum Trow and P. vanterpoolii V. and H. Kouyeas were identified by the use of the taxonomic monograph. The identified isolates were:

Pythium pythioides - 88-1-8 and 88-1-13: Colonies on cma with a chrysanthemum appearance. Zoosporangia globose, 9-24 µm diameter; smooth-walled; intercalary or terminal. Encysted zoospores globose, 9-10 µm diameter; motile zoospores pyriform to oval, 9 µm long x 6 µm wide. Oogonia globose, 21-24 µm diameter; smooth-walled; terminal or intercalary. One to two antheridia per ocgonium; antheridia diclinous, swollen-tipped. One oospore per oogonium; oospores reticulate; aplerotic; globose, 18-21 µm diameter; oospore wall 3-5 µm thick (figure III-1).

Pythium rostratum 88-1-5: Colonies on cma with a chrysanthemum appearance. Sporangia globose, 9-21 µm diameter; limoniform, 12-21 µm long x 9-15 µm wide;

intercalary or terminal. Oogonia globose, 15-24 µm diameter; smooth-walled; intercalary. One antheridium per oogonium; antheridia monoclinous, diclinous, or hypogynous. One oospore per oogonium; oospores plerotic; globose, 15-24 µm diameter; oospore wall 3 µm thick (figure III-2).

P. ultimum F41.1.b. Colonies on cma with a coarsely radiate appearance. Asexual structures globose, 20-26 µm diameter; limoniform, 24-36 µm long x 21 µm wide; smooth-walled; terminal or intercalary. Oogonia globose, 19-25 µm diameter; smooth-walled; terminal, intercalary. One to three antheridia per oogonium; antheridia hypogynous, diclinous; sac-like at tips. One oospore per oogonium; oospores aplerotic; globose, 15-21 µm diameter; smooth-walled; oospore wall 2-3 µm thick (figure III-3).

P. vanterpoolii 88-3-6. Colonies on cma with a mixed chrysanthemum, rosette appearance. Sporangia filamentous; inflated; smooth-walled; terminal. Oogonia globose, 13-20 µm diameter; smooth-walled; intercalary. One to four antheridia per oogonium; antheridia mostly monoclinous, also diclinous. One oospore per oogonium; oospores plerotic; globose, 13-19 µm diameter; smooth-walled; oospore wall 3-4 µm thick (figure III-4).

Other Pythium spp. isolates identified by the brc, were P. paroecandrum, P. sylvaticum and Pythium sp. Group G. Pythium rostratum was identified by both the brc and the taxonomic monograph. The identified isolates were:

Pythium paroecandrum F1.3.8 and F42.2.5. Sporangia globose, 12-33 µm diameter; smooth-walled; intercalary or terminal. Oogonia globose, 17-24 µm diameter; smooth-walled; intercalary in chains. One to three antheridia per oogonium; antheridia monoclinous, diclinous or hypogynous. One oospore per oogonium; oospores aplerotic; globose, 15-21 µm diameter; smooth-walled; oospore wall 1-2 µm thick (figure III-5).

Pythium rostratum F41.1.a. See above description.

Pythium sylvaticum F26.4.2 and F28.2.2. Hyphal swellings globose or limoniform, up to 32 µm diameter; intercalary or terminal. Oogonia globose, 18-20 µm diameter; smooth-walled; intercalary or terminal. Two to four antheridia per oogonium; antheridia diclinous; inflated. One oospore per oogonium; oospores aplerotic; globose, 15-18 µm diameter; smooth-walled; oospore wall 1-2 µm thick (figure III-7).

Pythium sp. Group G F70.1.b. Oogonia absent in cultures. Hyphal swellings present or absent (figure III-6).

Testing pathogenicity of Pythium spp. and other phycomycetes toward canola

i) wa plate method

Table III-1 shows results from the wa plate method to study the pathogenicity of 35 phycomycete isolates (including 16 Pythium spp.) in causing CSB of B. campestris cv. Tobin. This wa plate method focussed on the effect of isolates on seed germination. One of these isolates P. ultimum, was supplied by Dr. P. Verma of Agriculture Canada in Saskatoon, Saskatchewan.

Tobin seed germination was 98% in the absence of any fungus (control) (Table III-1). Germination of seed exposed to 12 of the isolates, was not significantly different from that of the control treatment. Seed germination ranged from 88-100% in the presence of these isolates. Twenty-three isolates resulted in a significant reduction in seed germination compared to that of the control treatment. Seed germination ranged from 0-84% in the presence of these isolates. A total of 19 isolates resulted in less than 50% seed germination. Of the identified isolates, P. paroecandrum F1.3.8, and P. ultimum F33.1.3, were the most virulent, both resulting in 0% seed germination. P. sylvaticum F26.4.2 and F28.2.2; P. ultimum Sask. and F41.1.b; and P. pythioides 1-13, all had statistically similar effects on seed germination as P. paroecandrum F1.3.8.

The germinated seedlings from the control treatment, had hypocotyls that were whitish-green in color, roots of uniform diameter and the absence of discoloration or lesions on any parts (figure III-8 A). Canola seedling blight symptoms were present when seed was exposed to 26 of the 35 isolates. These symptoms included non-germinated seed being soft; hypocotyls and roots being stunted, discolored, and containing lesions; and cotyledons containing dark brown necrotic dot lesions (figure III-8 B). Many of the isolates had extensive mycelial growth surrounding the seed and seedlings on the agar surface.

The six P. pythioides isolates resulted in seed germination of 12-68%, with all giving rise to CSB symptoms. The three P. paroecandrum isolates resulted in seed germination of 0-26%, with two isolates producing CSB symptoms. The two P. sylvaticum isolates resulted in seed germination of 4-8%, with both causing CSB symptoms. The three P. ultimum isolates resulted in seed germination of 0-6%, with all causing CSB symptoms. The Pythium sp. Group G, P. rostratum and P. vanterpoolii isolates resulted in seed germination of 38%, 88% and 96%, and the presence, absence and presence of symptoms, respectively.

ii) soil pathogenicity tests

Pathogenicity was determined for a total of 13 Phycomycete isolates, in causing CSB in soil. Results of pathogenicity testing of nine isolates to B. campestris cv. Candle are shown in Table III-2. There was 90% emergence in the control treatment. Eight isolates: P. paroecandrum F42.2.5, P. sylvaticum F26.4.2 and F28.2.2, P. ultimum F33.1.3 and F41.1.b, Pythium sp. Group G F70.1.b, and isolates F6.2 and F66.2.2 all resulted in significant reductions in percentage seedling emergence compared to the control treatment. These isolates resulted in percentage seedling emergence ranging from 55 to 1.3%. One isolate F53.2.1, had no significant effect on seedling emergence compared to that for the control.

In the control treatment, no seedlings had CSB symptoms (Table III-2). All isolates except F53.2.1, resulted in a significant increase in percent of seedlings with CSB symptoms, compared to that of the control. More than 50% of the seedlings had CSB symptoms when exposed to seven isolates F41.1.b, F6.2, F26.4.2, F66.2.2, F28.2.2, F42.2.5 and F33.1.3. Four of these F66.2.2, F28.2.2, F42.2.5 and F33.1.3, resulted in more than 90% of the emerged seedlings having CSB symptoms. These four isolates also resulted in less than 20% seedling emergence. Isolates F53.2.1 and F70.1.b, resulted in less than 30% of the seedlings having CSB symptoms. Table III-2 shows a general trend of certain isolates including P. sylvaticum F28.2.2, P. ultimum F33.1.3, P. paroecandrum F42.2.5 and isolate F66.2.2 resulting in both low levels of emergence as well as high levels of seedlings having CSB symptoms.

Results of pathogenicity testing of four Pythium spp. isolates to B. campestris cv. Tobin are shown in Table III-3. There was 90.4% emergence in the control treatment. The two identified P. pythioides isolates, 88-1-8 and 88-1-13 both resulted in significant reductions in percentage seedling emergence compared to the control treatment. These isolates resulted in 33.2% and 11.6% emergence, respectively. The other two isolates 88-1-5 and 88-3-6, identified as P. rostratum and P. vanterpoolii, respectively, had no significant effect on seedling emergence compared to the control.

In the control treatment, no seedlings had CSB symptoms (Table III-3). Both P. pythioides isolates 88-1-13 and 88-1-8, resulted in a significant increase in seedlings with CSB symptoms (more than 50% seedlings with CSB symptoms, for each isolate) compared to the control. The P. rostratum and P. vanterpoolii isolates 88-1-5 and 88-3-6, respectively, had no significant effect on percent of seedlings with CSB symptoms. These two isolates resulted in less than 5% of the seedlings having CSB symptoms. Table III-3 shows that certain isolates such as 88-1-8 and 88-1-13, resulted in low levels of emergence as well as high levels of seedlings having CSB symptoms.

Ten of a total of 13 tested phycomycete fungi were found to be pathogenic to B. campestris cvs. Candle and Tobin based on results from soil pathogenicity trials (Tables III-2 and III-3). These 10 fungi included a number of Pythium spp. isolates: P. pythioides 88-1-8 and 88-1-13, pathogenic to Tobin; and P. paroecandrum F42.2.5, Pythium species group G F70.1.b, P. sylvaticum F26.4.2 and F28.2.2, P. ultimum F33.1.3 and F41.1.b, and the isolates F6.2 and F66.2.2, all pathogenic to Candle. These 10 isolates resulted in significantly reduced percent emergence of seedlings and significantly increased percent seedlings with CSB symptoms, compared to the control treatment with no fungus added to the soil.

Both the wa plate and soil pathogenicity methods were employed to study the pathogenicity of four Pythium spp. isolates in causing CSB of the cv. Tobin. To determine whether results from these two methods followed similar trends, a comparison was made between the % germination and % emergence data (Tables III-1 and III-3 respectively). Table III-4 summarizes the results of pathogenicity testing of these four Pythium spp. isolates in causing CSB of Tobin, on water agar and in soil. The same general trend was observed from the wa and the soil pathogenicity testing of the four Pythium spp. isolates in causing CSB of B. campestris cv. Tobin (Table III-4). Infestation of soil and inoculation of water agar with isolates 88-1-5 or 88-3-6 did not significantly affect % emergence and % germination, respectively, compared to these results for the controls. Infestation of soil and inoculation of water agar with isolates 88-1-8 or 88-1-13 resulted in significant reductions in % emergence and % germination, respectively, compared to these results for the controls. Pythium pythioides, 88-1-13, resulted in the lowest percent seed germination, which was significantly different from all other isolates.

The CSB symptoms included hypocotyls with constrictions, tapering and brown necrotic regions; radicles being stunted; cotyledons with dark brown necrotic dot lesions; and non-germinated seed being soft and mushy. All isolates were re-isolated

from the tissues with CSB symptoms including cotyledons with dot lesions, as evidenced by fast-growing colonies consisting of aseptate hyphae. The control seedlings had hypocotyls that were thick and greenish-white, root systems being extensive, cotyledons lacking dot lesions and seed being firm.

One distinctive symptom of infection by Pythium spp. was the presence of dark brown necrotic dot lesions (figure 111-8 B). These dot lesions were present on cotyledons and upper portions of hypocotyls of seedlings exposed to various Pythium spp. isolates, in both the wa plate and soil pathogenicity experiments. The dot lesions varied in size, were usually dark brown in color and were present on both surfaces of the cotyledons. Dot lesions were absent from cotyledons of seed not exposed to Pythium spp. (figure 111-8 A).

To determine whether Pythium spp. were associated with these dot lesions, small pieces of cotyledons containing single lesions were excised, surface disinfected, rinsed and plated. Cotyledons from seed not exposed to Pythium spp. were likewise treated. Pythium spp. isolates were recovered from cotyledon pieces containing dot lesions. No Pythium spp. isolates grew from cotyledon pieces which had no dot lesions. These results indicate that one symptom of infection of canola by Pythium spp. is the presence of dark brown necrotic dot lesions on cotyledons and upper portions of hypocotyls. Pythium spp. were recovered from tissues surrounding these lesions.

D. Discussion

Other studies have shown that fungal pathogens causing damping-off can be isolated by the use of seed 'bait'. Seed of onion, lettuce, chickpea and cucumber were colonized by a number of Pythium spp. including P. aphanidermatum (Edson) Fitz., P. paroecandrum, P. sylvaticum and P. ultimum after being placed into field soils (2,16,23). In this study, seed of B. campestris cv. Candle were used as bait to obtain Pythium spp. involved in CSB and especially its pre-emergence phase. A total of 40.6% of the Candle seeds were

colonized by Pythium spp. and other Phycomycetes within three days after the seeds were placed into various soils. Pythium paroecandrum, P. pythioides, P. rostratum, P. sylvaticum, P. ultimum, P. vanterpoolii, and Pythium sp. Group G. all colonized cv. Candle seed. Although P. ultimum was previously reported as being isolated from rapeseed plants (18), this is the first report of these seven Pythium spp. being isolated from canola seed.

Pythium pythioides 88-1-8 and 88-1-13, were identified as P. coloratum Vaartaja by the brc, and P. dissimile Vaartaja by the cmi. Pythium rostratum 88-1-5 and P. vanterpoolii 88-3-6 were identified as P. salpingophorum Drechsler and P. pleroticum T. Ito, respectively, by the brc.

Identification of isolates 88-1-8 and 88-1-13 as P. pythioides, was based upon the appearance of oospores which appeared to be reticulate and aplerotic. The oogonial position and size, the numbers of antheridia attached to each oogonium and the development of the antheridia and their shape all matched the descriptions for this species given in the taxonomic monograph (21). In addition, motile zoospores were seen. These were of a similar shape and size as those descriptions given for these in the taxonomic monograph. The presence of reticulate oospores was the most important feature used in the identification of these two isolates as P. pythioides. Isolate 88-1-5 was identified as P. rostratum. All characteristics of this isolate matched those given in the taxonomic monograph. Three important features used in the identification of 88-1-5 as P. rostratum, were the presence of intercalary oogonia which have been reported to be often catenulate (21), hypogynous antheridia and the reported isolation of P. rostratum from the roots of a number of plants but the non- or low virulent nature of this species (21). This reported degree of low virulence matches that obtained in results from the pathogenicity trials of 88-1-5 on canola (Tables III-1 and III-3). Two isolates F33.1.3 and F41.1.b, were identified as P. ultimum. Pythium ultimum has two varieties, var. ultimum and var. sporangiiferum (21). Distinctions between these two are based on the

presence of hyphal swellings with rarely produced sporangia and zoospores for P. ultimum var. ultimum, or presence of sporangia producing zoospores at room temperature for P. ultimum var. sporangiiferum. The observed asexual structures for both isolates were similar in size to those of hyphal swellings. Based on the size of the asexual structures and the absence of zoospores at room temperature, both isolates F33.1.3 and F41.1.b are probably P. ultimum var. ultimum. Isolate 88-3-6 was identified as P. vanterpoolii. The primary reason for this identification is the presence of filamentous inflated sporangia. These distinctive sporangia and all other observed characteristics matched those given in the taxonomic monograph (21). The reported degree of low virulence for P. vanterpoolii (21) agrees with the finding of non-pathogenicity of 88-3-6 in causing CSB (Tables III-1 and III-3).

Pathogenicity of the various Pythium spp. and other Phycomycetes in causing CSB was determined by studies of seed germination on agar plates and seedling emergence from soil. Seed germination and seedling emergence studies provided information concerning pre-, and both pre- and post-emergence damping-off, respectively, which are the two aspects of CSB (20).

Sixteen identified Pythium spp. isolates (this number excludes the P. ultimum isolate obtained from Saskatchewan) were tested for their effects on germination of Tobin seed (Table III-1). Reduced seed germination is one aspect of CSB. Fourteen of these isolates were found to be important in causing CSB, because of their effects on seed germination. These isolates which included P. paroecandrum (3 isolates), P. pythioides (6 isolates), P. sylvaticum (2 isolates), P. ultimum (2 isolates) and Pythium sp. group G (1 isolate), all significantly reduced germination of Tobin seed compared to controls (Table III-1). Thirteen of these isolates reduced seed germination to below 50% compared to 98% seed germination for controls. A reduced level of seed germination resulting from infection by Pythium spp., will translate to decreased stand establishment. All these isolates resulted in CSB symptoms on germinated seedlings, with

the exception of one isolate of P. paroecandrum which resulted in all seed not germinating.

Differences existed amongst the tested isolates of each Pythium sp., concerning their effects on seed germination (Table III-1). The three P. ultimum isolates resulted in 0 to 6% seed germination. The two P. sylvaticum isolates resulted in 4 and 8% seed germination. The three P. paroecandrum isolates resulted in 0 to 26% seed germination. The six P. pythioides isolates resulted in 12 to 68% seed germination. This result of differences in % germination of Tobin seed when exposed to various isolates of the same Pythium sp. indicates that different isolates of the same Pythium sp. have differences in virulence in causing CSB.

Both P. rostratum 88-1-5 and P. vanterpoolii 88-3-6 were shown to be non-pathogenic to Tobin based on results of seed germination on wa (Table III-1) and seedling emergence from soil (Table III-3). The effects of each of these two isolates on seed germination, seedling emergence and % of seedlings with CSB symptoms, were not significantly different from controls. Only P. vanterpoolii resulted in some CSB symptoms on germinated Tobin seed. Neither of these isolates resulted in more than 3% of the seedlings having CSB symptoms.

Infection of seedlings before, or after their emergence from the soil may result in post-emergence damping off (1). Pythium paroecandrum, P. pythioides, P. sylvaticum, P. ultimum, and Pythium sp. Group G all infected B. campestris cv. Tobin and resulted in CSB-symptoms on seed and seedlings (Table III-1). These Pythium spp. are therefore potentially involved in the post-emergence damping-off phase of CSB.

Both the pathogenicity tests concerning seed germination on wa and seedling emergence from soil, yielded similar results for the identified Pythium spp. Pythium paroecandrum, P. pythioides, P. sylvaticum, P. ultimum, and Pythium sp. Group G were all found to be pathogenic in causing CSB of B. campestris cvs. Candle and Tobin, based on

results of seed germination (Table III-1) and seedling emergence (Tables III-2 and III-3).

All Pythium spp. isolates were not tested at the same inoculum (cfu) level in the soil pathogenicity tests. Data were compared between the control treatments and each of the isolates, and means were separated by the Least Significant Difference test because of the non-uniformity of inoculum levels used. No comparisons were made amongst the isolates.

As in the results from the testing of seed germination on wa, differences existed between the tested isolates of each of P. pythioides, P. sylvaticum and P. ultimum concerning their effects on seedling emergence and emerged seedlings having CSB symptoms (Tables III-2 and III-3). Differences in inoculum levels and virulence may account for these differences in results between isolates of the same Pythium sp.

Pythium sylvaticum F28.2.2 while being infested into soil at a 13% lower inoculum level than P. sylvaticum F26.4.2, resulted in a far greater effect on reduction of seedling emergence and increase in the percent of seedlings with CSB symptoms (Table III-2). This suggests that F28.2.2 is more virulent than F26.4.2. Pythium ultimum F33.1.3 which was infested into soil at a 13% higher inoculum level than P. ultimum F41.1.b, also resulted in a far greater effect on reduction of seedling emergence and increase in the % of seedlings with CSB symptoms (Table III-2). This greater pathogenic effect of F33.1.3 than F41.1.b was due to the differences in inoculum levels and a possible difference in virulence between these two isolates.

Any additional pathogenicity testing of Pythium spp. in soil should involve the same inoculum level for all isolates. This would allow for comparisons to be made amongst all isolates of the same species and amongst different species. This would also allow for comparisons to be made between results of seedling emergence in soil and seed germination on agar.

Beetle feeding damage has been associated with necrotic spots on cotyledons of canola seedlings (Alberta Agriculture, 'Canola seedling damage' poster). The pathogenicity tests on wa plates and in soil, showed that one symptom of infection by Pythium spp. isolates was the presence of dark brown, necrotic dot lesions on seedling cotyledons. These lesions were absent on seedlings not exposed to Pythium spp. These dot lesions are a result of Pythium infection as evidenced by recovery of Pythium from cotyledon tissues containing single lesions and no recovery of Pythium from cotyledons of seedlings not exposed to Pythium and lacking these lesions. Presence of necrotic spot lesions on red clover cotyledons, has been reported elsewhere (10). Cotyledon dot lesions were observed during collection of canola field seedlings with CSB symptoms. This is the first known report of the presence of these cotyledon dot lesions on canola seedlings, and the association of these lesions with Pythium spp. infection. The significance of this finding is that dot lesions on young canola field seedlings may be the result of beetle feeding and/or infection by Pythium spp.

Table III-1. Pathogenicity of Phycomycetes, including Pythium spp. in causing CSB of B. campestris cv. Tobin

isolate	spec+	% G[x]		sym[s]	isolate	spec+	% G[x]		sym[s]
F33.1.3	Pu	0	a	+	1-11	Py	40	g	+
F1.3.8	Pp	0	ab	n/a	F53.2.1	-	62	h	+
F26.4.2	Ps	4	ab	+	1-8	Py	68	h	+
P.ult	Sa	4	abc	+	4-5	-	76	hi	+
F41.1.b	Pu	6	abc	+	5-10	-	84	hij	+
F28.2.2	Ps	8	abcd	+	1-5	Pr	88	ijk	-
F6.2	-	10	bcde	+	2-8	-	90	ijk	+
1-13	Py	12	bcde	+	1-1	-	92	ijk	-
F66.2.2	-	12	bcde	+	3-10	-	92	jk	+
1-4	-	14	bcde	+	5-3	-	94	jk	+
F42.2.5	Pp	14	cdef	+	1-20	-	94	jk	-
1-16	Py	22	defg	+	2-12	-	96	jk	-
1-10	Pp	26	defg	+	3-6	Pv	96	jk	+
1-6	-	28	efg	+	1-2	-	96	jk	-
1-21	Py	36	fg	+	1-19	-	96	k	-
5-1	-	36	fg	+	CONTROL	-	98	k	-
1-12	Py	36	fg	+	1-9	-	98	k	-
F70.1.b	PG	38	g	+	1-23	-	100	k	-

[x] % germination, means based on 5 replicate petri plates; isolates with same letter are not significantly different at P=0.05 according to Duncan's multiple range test; letters from transformed data

[+] species, codes: PG, Pythium species Group G; Pp, P. paroecandrum; Pr, P. rostratum; Ps, P. sylvaticum; Pu, P. ultimum; P.ult Sa, P. ultimum from Sask.; Pv, P. vanterpoolii; Py, P. pythioides; -, not completed, or unknown identification

[s] symptoms:
+ presence of CSB symptoms on most seed/seedlings
- absence of CSB symptoms on most seed/seedlings

Table III-2. Pathogenicity of nine Phycomycete isolates in causing CSB of B. campestris cv. Candle

Treatment[P]	% emergence[+]	% seedlings with CSB symptoms[S]
F53.2.1	92.5	4.0
Control[0]	90.0	0.0
F41.1.b	55.0 *	64.3 *
F26.4.2	50.0 *	79.3 *
F6.2	48.8 *	76.0 *
F70.1.b	40.0 *	29.4 *
F28.2.2	16.3 *	95.8 *
F66.2.2	7.5 *	91.7 *
F33.1.3	3.8 *	100.0 *
F42.2.5	1.3 *	100.0 *

P F33.1.3, F41.1.b, P. ultimum; F26.4.2, F28.2.2, P. sylvaticum; F42.2.5, P. paroecandrum; F70.1.b, Pythium sp. Group G; F6.2, F53.2.1, and F66.2.2 not identified

Inoculum levels, cfu ($\times 10^4$)/ml: F6.2, 68; F26.4.2, 77; F28.2.2, 67; F33.1.3, 68; F41.1.b, 60; F42.2.5, 137; F53.2.1, 19; F66.2.2, 117; F70.1.b, 98

0 non-infested soil

+ means based on 8 replicate pots

S means based on only those replicate pots with emerged seedlings

* significantly different from control, at P=0.05, according to LSD test on transformed data

Table III-3. Pathogenicity of four Pythium spp. isolates in causing CSB of B. campestris cv. Tobin

Treatment[P]	% emergence[+]	% seedlings with CSB symptoms[S]
88-1-5	91.6	2.3
Control[0]	90.4	0.0
88-3-6	82.0	0.6
88-1-8	33.2 *	58.9 *
88-1-13	11.6 *	67.6 *

[P] 88-1-8, 88-1-13, P. pythioides; 88-1-5, P. rostratum; 88-3-6, P. vanterpoolii
Inoculum levels, cfu (x 10^4)/ml:
88-1-5, 32; 88-1-8, 42; 88-1-13, 184; 88-3-6, 132

[0] non-infested soil

[+] means based on 8 replicate pots

[S] means based on only those replicate pots with emerged seedlings

* significantly different from control, at P=0.05, according to LSD test on transformed data

Table III-4. Pathogenicity of four Pythium spp. isolates in causing CSB of B. campestris cv. Tobin; combined results from wa plate+ and soil studies#

Treatment	% Emergence#		% Germination+	
88-1-5	91.6		88	a
Control	90.4		98	a
88-3-6	82.0		96	a
88-1-8	33.2	*	68	b
88-1-13	11.6	*	12	c

\+ from Table III-1; values with same letters are not significantly different at P=0.05 according to Duncan's multiple range test; letters from transformed data

\# from table III-3

* significantly different from control at P=0.05, according to LSD on transformed data

Figures III-1 to III-7 showing Pythium spp. which colonized Brassica campestris cv. Candle seed.

Figure III-1. (upper). Pythium pythioides.

(A). Globose oogonium containing one aplerotic reticulate oospore.

(B). Intercalary globose sporangium.

Figure III-2. (lower). Pythium rostratum.

(A). Intercalary globose oogonium containing one plerotic oospore.

(B). Intercalary limoniform sporangium.

A
B
A
B

Figure III-3. (upper). Pythium ultimum.

(A). Empty hooked antheridium attached to globose oogonium containing one smooth-walled aplerotic globose oopore.

(B). Intercalary globose asexual structure.

Figure III-4. (lower). Pythium vanterpoolii.

(A). Terminal globose oogonium containing one plerotic oospore.

(B). Filamentous inflated sporangia.

A
A
B

Figure III-5. (upper). Pythium paroecandrum.

(A). Globose oogonium containing one aplerotic globose oospore.

(B). Terminal globose sporangium.

Figure III-6. (lower). Pythium sp. Group G.

(A and B). Intercalary and terminal globose hyphal swellings, respectively.

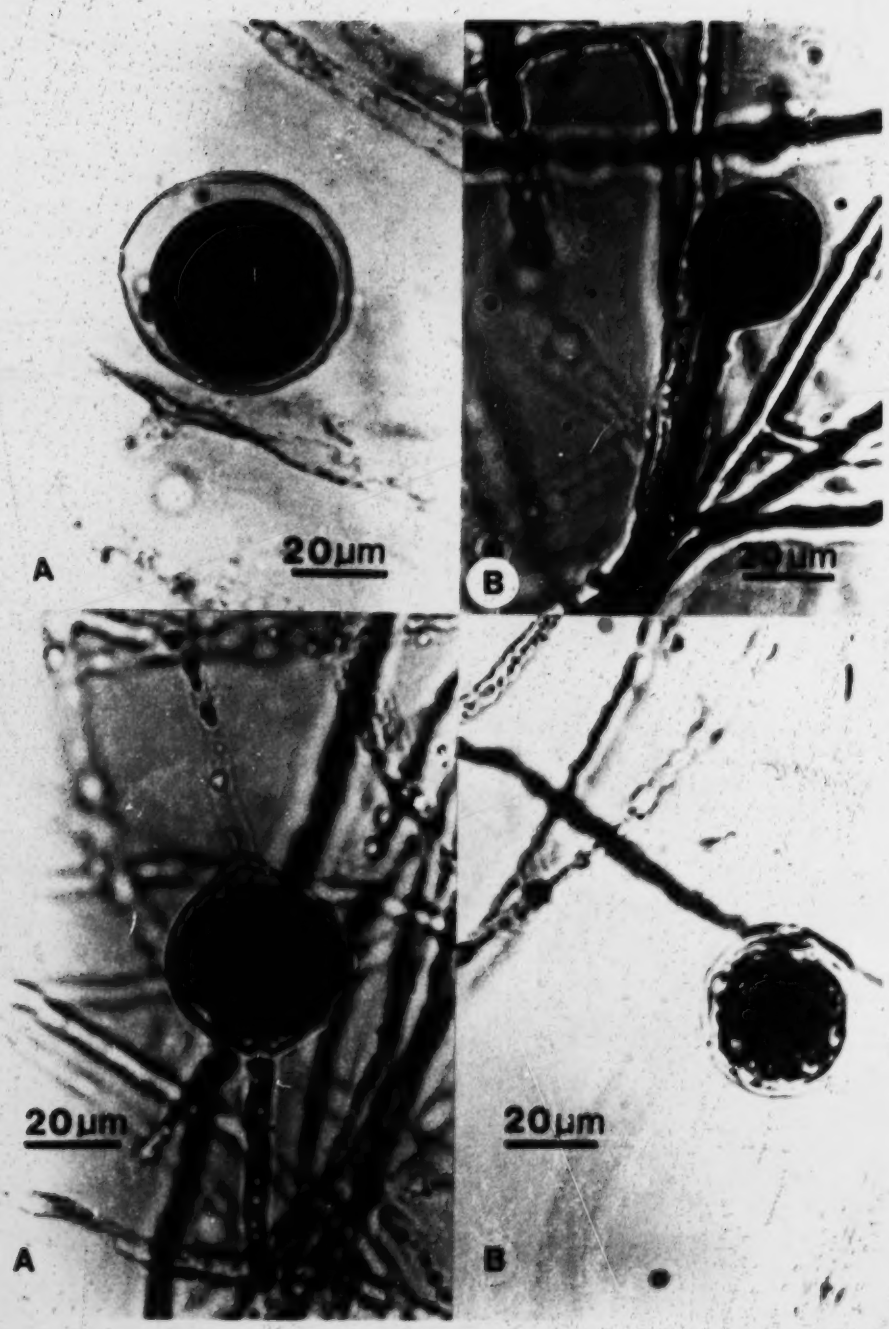

A
20µm
B
20µm
20µm
A
20µm
B

Figure III-7. Pythium sylvaticum.

(A). Intercalary limoniform hyphal swelling.

(B). Young globose oogonium and surrounding antheridia.

(C). Empty antheridium attached to globose oogonium containing one aplerotic globose oospore.

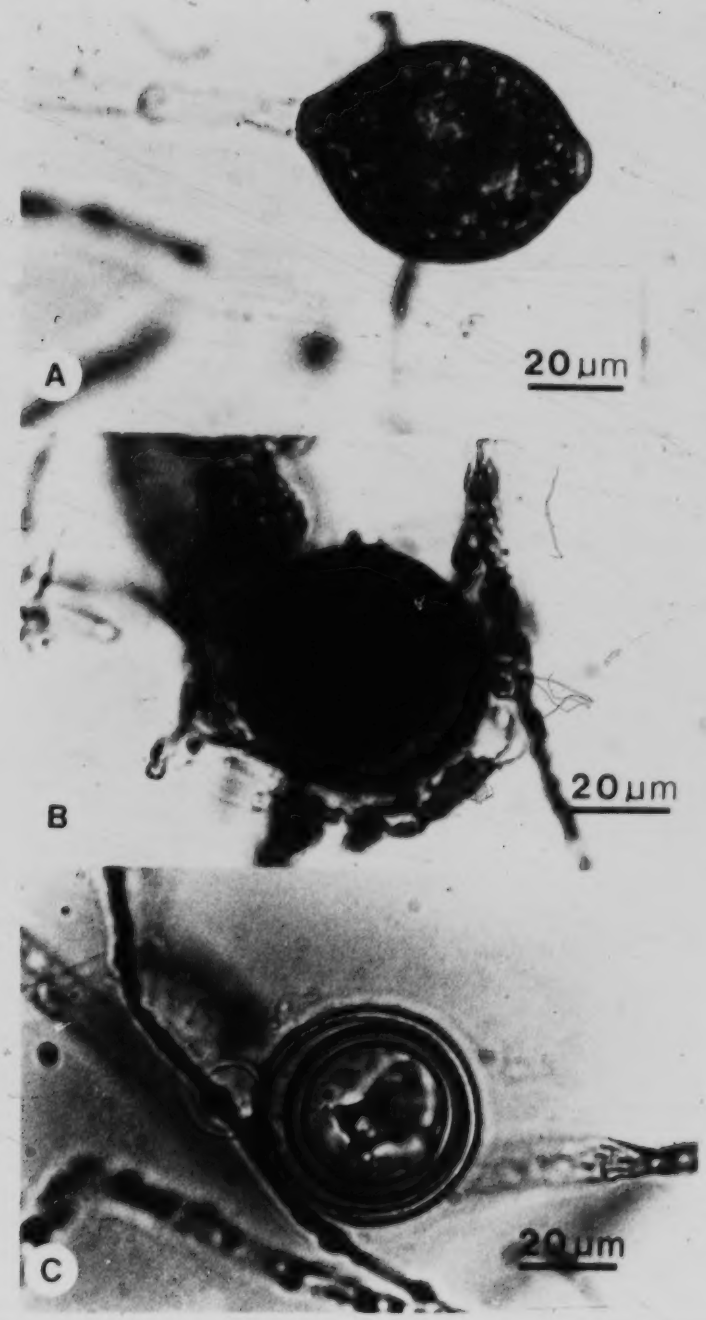
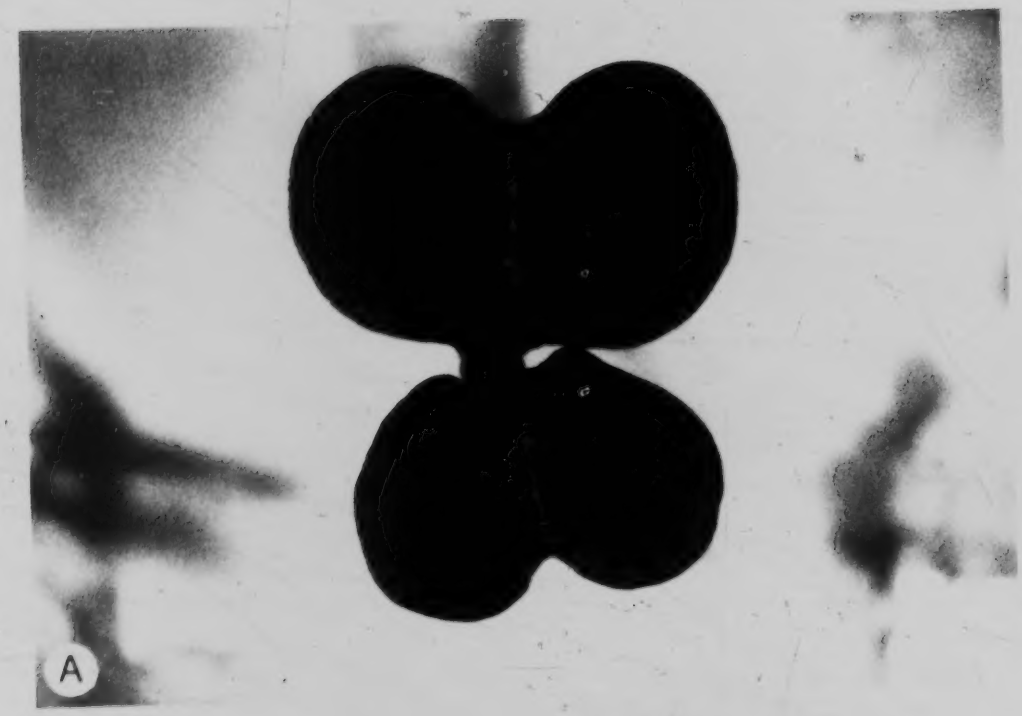
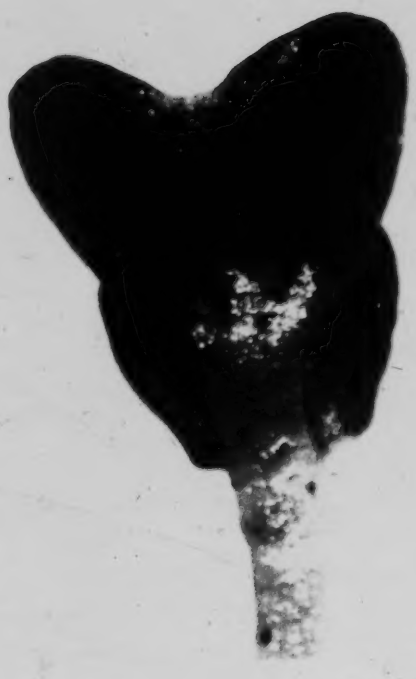

A
20 µm
B
20 µm
C
20 µm

Figure III-8. Cotyledons of Brassica campestris cv. Tobin.

(A): Top view of cotyledons from seed not exposed to Pythium sp.

(B). Side view of dot lesions on cotyledons resulting from seed infection by Pythium pythioides.





B

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IV. Biological control of R. solani caused canola seedling blight

A. Introduction

Rhizoctonia was the most frequently isolated fungus genus from canola/rapeseed (canola) field seedlings with CSB symptoms (Chapter II, Table II-1). A total of 33.8% of all fungal isolates were Rhizoctonia spp. Rhizoctonia solani was shown to be highly virulent in causing CSB (Chapter II, Tables II-2 and II-3).

Recommended control practices for CSB include crop rotation, control of volunteer canola and weeds, planting certified seed at depths of less than 2.5 cm into firm moist warm seed beds and use of chemical seed treatments. Chemicals recommended for seed treatment include Benolin (benomyl), Rovral (iprodione), Vitavax (carbathiin), and Thiram (18). Two experimental fungicides for control of CSB are metalaxyl which is effective against Pythium spp. and rizolex which has been reported to be effective against R. solani (1; and Gabrielle, K. 1985. personal communication, respectively).

Biological control involving the use of non-pathogenic microorganisms, offers another means of controlling seedling pathogens. A number of Trichoderma spp. have been reported to be effective bio-control agents for seedling diseases. Trichoderma hamatum (Bonord.) Bain. was found to reduce the amount of damping-off of pea and radish caused by Pythium spp. and R. solani, respectively (8). Damping-off of bean, eggplant and tomato in R. solani-infested soil was controlled by T. harzianum Rifai (7). Both T. harzianum and T. koningii Oudem. reduced the amount of pre-emergence damping-off of pea in a Pythium sp.-infested soil (14).

Experiments were undertaken to determine whether CSB caused by R. solani, can be controlled by Trichoderma spp.

B. Materials and Methods

Collection of soils for isolation of Trichoderma spp.

A total of 47 different soils collected from from poplar and spruce forests, and fields planted to canola, barley and wheat from throughout Alberta, were sources of Trichoderma spp. Soils were collected in August/September, 1985, and June, 1986, from southern Alberta around Lethbridge, central Alberta around Edmonton, and northern Alberta around Fairview and High Level (figure IV-1). Soil from each site was collected by the same procedure as that described for collection of diseased field seedlings (Chapter II, Materials and Methods), bulked together and processed within four to 17 days.

Isolation of Trichoderma spp. potentially antagonistic to R. solani

Three methods were used for isolation of Trichoderma spp.: i.) selecting colonies from soil dilutions that were plated on an acidified potato dextrose agar medium (apda) for general growth of fungi and a Trichoderma-selective medium (tsm) (16); ii.) use of a modified soil baiting method (21); and iii.) use of a triple agar layer technique (10).

i.) selecting colonies from tsm and apda

Colonies from these media were transferred to 1.5% water agar (wa) plates. Colony margins were then transferred from the colonies on wa, to potato dextrose agar (pda) plates. All plates were incubated at room temperature and room light (rtl). Colonies were identified as Trichoderma spp. based on their rapid spreading growth producing green/white aerial pustules, regularly septate hyphae, aerial loosely arranged conidiophores bearing one-celled conidia from phialides and sometimes bearing sterile appendages. Pure Trichoderma spp. colonies having uniform growth on pda, were then transferred to pda slants and stored at 4^{o}C.

ii.) modified soil baiting technique

Rhizoctonia solani was grown in still cultures of Czapek-Dox broth for five days at rtl. The fungus mycelium was collected and placed onto a sterilized Nitex® screen (with1 mm^2 openings) which was pressed into field soil (moistened with sterile tap water) inside each of three 15 cm diameter initially sterilized glass petri plates. The plates were closed and incubated at 16°C in the dark for three days. Rhizoctonia solani mycelium was then removed from the screen, washed under running tap water to remove excess soil debris and pieces were placed onto plates of tsm, apda, Richard's medium (rm) and dextrose peptone-rose bengal (dp) (20). The plates were incubated and colonies were selected.

iii.) triple agar layer technique

This technique involved production of three agar layers in 10 cm diameter sterile petri plates. These were a lower 10 ml layer of 1.5% water agar (wa), a middle 5 ml layer of wa containing a 1/4000 soil dilution and an upper 5 ml layer of Czapek-Dox agar containing macerated R. solani mycelium. The soil dilution layer was incubated on the lower wa for two days at rtl before addition of the upper layer. Rhizoctonia solani was grown in Czapek-Dox broth for three days at rtl, macerated in a Waring blendor and mixed with an equal volume of molten sterile Czapek-Dox agar. The Czapek-Dox agar layer containing macerated R. solani was then added to the soil dilution layer. The 1/4000 dilution of each soil and upper R. solani layers were incorporated into five replicate petri plates. The plates were incubated for three days at rtl. Numbers of inhibition zones were counted in the upper R. solani-Czapek-Dox layer and transfers were then made from the central region of each inhibition zone to pda plates. Margins of colonies developing on pda, were transferred to apda to eliminate possible bacterial contaminants. Colony margins were then transferred from apda to pda plates and then to pda slants on which pure colonies were stored, at 4°C.

Selection of Trichoderma spp. showing most promising antagonistic effects toward R. solani

Two types of studies were carried out to select Trichoderma spp. isolates showing the most promising antagonistic effects toward R. solani: i.) observations of colony interactions between R. solani and various Trichoderma spp. (19), and ii.) observations of hyphal interactions between R. solani and various Trichoderma spp. (11).

i.) observations of colony interactions between R. solani and Trichoderma spp.

Visual observations of specific colony interactions were made between pairings of a seedling blight causing isolate of R. solani and one of a number of Trichoderma spp. isolates. Fungi were individually grown on pda for four days at rtl. One five mm diameter plug from the colony margin of each of the two fungi involved in the pairing was placed on a pda plate, with the two paired plugs being placed about four cm apart. Each pair of plugs was placed onto three to five replicate plates. For controls, each fungus was grown separately on two to five pda plates. Plates were incubated for a minimum of three days at rtl, after which time the colony interactions were recorded. The following colony interactions were recorded:

MI - mutual intermingling of hyphae in merging regions between the two colonies

OT - overgrowth by test fungus (each Trichoderma sp. isolate)

OH - overgrowth by bait fungus (R. solani)

S - sporulation by 'test' fungus in region where colonies merge (degree of sporulation was recorded)

ii.) observations of hyphal interactions between R. solani and Trichoderma spp.

Microscopic examinations were made of interacting hyphae of a seedling blight causing isolate of R. solani and a number of Trichoderma spp. isolates. Rhizoctonia solani and the various Trichoderma spp. isolates were first grown on pda plates for three to four days at rtl. Five mm diameter plugs were taken from colony margins of the various fungi grown on pda. Each pair of plugs, with individual plugs being separated by about three cm distance, was placed onto four sterilized cellophane strips on 1.5% wa plates. Controls consisted of five mm diameter plugs of the various fungi placed separately onto sterilized cellophane strips on wa plates. The inoculated wa plates were incubated at rtl for three to seven days. After this time, sections of cellophane containing merged hyphae of both fungi were cut, placed onto glass microslides, stained by flooding with lactophenol cotton blue and observed by light microscopy. Presence or absence and degree of the following specific phenomena were noted:

CL- coiling of Trichoderma spp. hyphae about hyphae of R. solani

PT- penetration of R. solani hyphae by hyphae of Trichoderma spp.

ST/A- swollen tips/appressoria of hyphae of Trichoderma spp. when contacting hyphae of R. solani

CT- cutting of R. solani hyphae in presence of hyphae of Trichoderma spp.

Control of R. solani in soil by coating canola seed with Trichoderma spp.

Two selected potential antagonists, N.45.7 and 85.12.2 applied as seed coatings, were tested to determine: 1.) whether they provided control of R. solani-caused CSB, and 2.) whether they were as effective as chemical seed treatment for control of this disease. These two isolates were chosen based on the colony and hyphal interactions observed between them and R. solani (Table IV-1).

Rhizoctonia solani C51.25, previously found to be pathogenic to canola was employed in these studies (Chapter II, Results). Inoculum consisted of dried ground rye seed on which the isolate had been grown (Chapter II, Materials and Methods).

Single spore cultures of both Trichoderma spp. isolates were grown on pda in two 10 cm diameter petri plates at rtl for seven days. Both fungi underwent intense sporulation under these conditions. Conidia of each isolate were collected by the following methods (14). Conidia were washed from the two plates in a total of 80 ml of sterilized distilled water, filtered through four layers of cheesecloth and centrifuged at 2500 g and 4°C for 15 minutes. The resulting conidial pellet was suspended in water and recentrifuged a total of four times. The conidia were suspended in a known volume of water and the number of conidia/ml was estimated using a haemacytometer. For coating seed, conidia were suspended in a sterile 1.5% (w/v) methyl cellulose suspension. The methyl cellulose was used to adhere the conidia to the seed.

Brassica campestris cv. Tobin seed was used in this study. All seed was surface disinfected by previously described methods (Chapter II, Materials and Methods). Five grams of surface disinfected Tobin seed (Tb) were thoroughly mixed with 4 ml of methyl cellulose suspension containing 3×10^8 conidia/ml, for each Trichoderma sp. isolate. The coated seed was then air-dried at room temperature and stored at 4°C until planted.

Viability of the conidia of the Trichoderma spp. isolates coated onto the Tobin seed was determined for seed that had been stored at 4°C for 12 days. Fourteen seeds coated with each of the two isolates were aseptically plated onto two pda plates which were incubated at rtl.

The various seed treatments used in this experiment were:

i) Tb.

ii) Tb coated with 1.5% methyl cellulose (Tb-mc).

iii) Tb coated with Trichoderma spp. N.45.7, or 85.12.2, suspended in 1.5% methyl cellulose (Tb-mcN, or Tb-mc85, respectively).

iv) Tobin commercially treated with Vitavax fungicide (Tobin-V).

v) Tb treated with an experimental fungicide mixture of Benlate, Metalaxyl, and rizolex (respective active ingredients/kg seed, of 1.25 g, 1.5 g, and 1.5 g) suspended in 1.5% methyl cellulose (Tb-ef).

The experiment was begun within one day after the seeds were coated with the two fungi. Each of the six seed treatments was planted in both non-infested soil and in R. solani-infested soil. Each treatment was replicated in six 9 cm x 9 cm pressed peat fiber pots containing 25 seeds per pot. Soil infestation with R. solani was at the rate of 0.35 g inoculum (61,560 cfu/g) per one kg soil (based on Chapter II, Results). Filling of pots including covering seeds with soil, watering of pots and type of data collected were previously described (Chapter II, Materials and Methods). The experiment was conducted as a completely randomized design, in one Conviron® growth cabinet set at 18°C day/7°C night, with a 16 h photoperiod (337 UE/m^2 sec). The experiment lasted 14 days. Data for % emergence and % seedlings with CSB symptoms were subjected to arcsin transformations prior to ANOVA, with means being compared by the Duncan's multiple range (dmr) test.

Control of R. solani in soil by the use of Trichoderma sp.-wheat bran amendments

Experiments were conducted to determine whether Trichoderma sp. colonized wheat bran amendments could control CSB in R. solani-infested soil. Some amendments included chitin since other studies have shown that the addition of chitin to growth solutions, stimulated chitinase production by Trichoderma spp. Chitinase production is associated with cell wall degradation of R. solani (4).

One Trichoderma sp. isolate 85.46.5a, was chosen for this experiment, based on the colony and hyphal interactions observed between it and R. solani(Table IV-1). This isolate grew over and underwent intense sporulation on colonies of R. solani. Hyphae of

this isolate underwent intense coiling around hyphae of R. solani and there was a great amount of cutting of R. solani hyphae in it's presence. This isolate has been identified as T. harzianum (brc).

One R. solani isolate C51.25, was employed in these studies. The inoculum consisted of dried ground rye seed on which the isolate was grown (Chapter II, Materials and Methods).

A single spore culture of T. harzianum 85.46.5a, was used to colonize wheat bran (wb). This isolate was grown on pda plates at rtl for eight days, during which time it underwent intense sporulation. A conidial suspension was obtained by flooding the plates with sterile distilled water. Three one l flasks, each containing 50 g dried wb moistened with 50 ml of distilled water (12), were sterilized, and then each flask was inoculated with about 10 ml of conidial suspension. All flasks were incubated with periodic shaking at rtl for 10 days. A small portion of the wheat bran overgrown by T. harzianum was suspended in sterile distilled water. This suspension was observed by light microscopy to determine the structures present.

The T. harzianum 85.46.5a-colonized wheat bran (wb-Tr) was air-dried in closed paper bags, passed through a 2 mm sieve and wb-Tr smaller than 2 mm in size was stored at rtl and used in the experiments. Number of viable propagules in the wb-Tr was determined by suspending 1 g of dried wb-Tr in a dilution series of 0.1% wa, plating 1.0 ml aliquots of chosen dilutions onto each of four pda plates and counting colonies after plates were incubated at rtl.

Brassica campestris cv. Tobin seed was used in this experiment. All seed was surface disinfected by previously described methods. Percent germination was determined for this seed. Fifty seeds were placed into each of four petri plates containing sterile moistened paper towelling. Numbers of germinated seed were counted after seed had been incubated for 72 h at room temperature in the dark.

One treatment in this experiment was:

i) surface disinfected seed (seed alone).

Each of the additional treatments included surface disinfected seed (seed alone) plus some amendment:

ii) seed alone plus uncolonized wheat bran (wb).

iii) seed alone plus chitin (ch) (chitin from crab shells, Sigma Chemical Co.).

iv) seed alone plus wb plus ch (wbch).

v) seed alone plus T. harzianum 85.46.5a-colonized wb (wbTr).

vi) seed alone plus wbTr plus ch (wbTrch).

The experiment involved each of the six treatments being planted in both non-infested soil and in R. solani-infested soil, and incubated under controlled conditions in a growth cabinet. All experimental methods were the same as those described for the experiment to control R. solani by coating seed with Trichoderma spp., with the seeds in each pot being covered with one of the above amendments at the rates of 1g/pot for wb, 1g/pot for wbTr and 0.08g/pot for ch and then covered to a depth of 2.5 cm with either non-infested soil or R. solani-infested soil.

C. Results

Isolation of Trichoderma spp. potentially antagonistic to R. solani

i.) selecting colonies from tsm and apda

A total of 119 different Trichoderma spp. isolates were obtained from soil dilutions of the 47 field soils collected in 1985 and 1986. Of all three methods, this technique yielded the greatest number of Trichoderma spp. isolates.

ii.) modified soil baiting technique

This technique involved the use of one R. solani isolate as a bait in one of the collected field soils. A total of 19 colonies grew from all pieces of R. solani bait hyphae

on the various media. Seventeen of these were R. solani and two colonies were mucoraceous fungi. No Trichoderma spp. isolates were obtained by the methods and conditions used in this technique.

iii.) triple agar layer technique

This technique was employed for two soils, using one R. solani isolate. Inhibition zones in the R. solani layer were difficult to detect, after three days incubation of all layers. A total of five inhibition zones (one from one soil, four from the second soil) were present in the R. solani layer. No Trichoderma spp. isolates were obtained by this technique.

Selection of Trichoderma spp. showing most promising antagonistic effects toward R. solani

i.) observations of colony interactions between R. solani and Trichoderma spp.

Colony interactions were studied between a total of 22 Trichoderma spp. isolates and R. solani. Included were one isolate each of T. harzianum and T. longibrachiatum Rifai, both of which were obtained from the University of Alberta Mold Herbarium(uamh) (specimen numbers 5069 and 5068, respectively) and one Trichoderma sp. isolate obtained from a coffee leaf (supplied by Dr. J. P. Tewari). Mutual intermingling of hyphae occurred between all 22 isolates and R. solani. Sixteen isolates including T. harzianum, overgrew R. solani. Rhizoctonia solani overgrew the paired colonies of three isolates including both T. harzianum and T. longibrachiatum. Trichoderma longibrachiatum neither grew over nor sporulated on the paired colony of R. solani. Nineteen isolates including T. harzianum, sporulated on the paired colony of R. solani. Very little differentiation could be made amongst the 22 isolates, based on the studied colony interactions.

ii.) observations of hyphal interactions between R. solani and Trichoderma spp.

Hyphal interactions were studied between a total of 24 Trichoderma spp. isolates and R. solani. Cutting of R. solani hyphae into hyphal fragments in the presence of various isolates and coiling of hyphae of the various isolates about R. solani hyphae were the two most frequently observed interactions. These two phenomena resulted during the interactions between 20 and 19 of the Trichoderma spp. isolates, respectively, and R. solani. Some isolates had hyphae that underwent highly intense coiling around R. solani hyphae and also that resulted in extensive cutting of hyphae of R. solani into a large number of hyphal fragments. One isolate 85.46.5a, whose hyphae showed intense coiling around and extensive cutting of R. solani hyphae, was identified as being T. harzianum (identified by Dr. J. Bissett of Agriculture Canada) (figure IV-2). Sixteen Trichoderma spp. isolates showed both coiling of hyphae around R. solani hyphae and cutting of R. solani hyphae in their presence. Hyphae with swollen tips/appressoria were observed during the interactions between two of the Trichoderma spp. isolates and R. solani. Hyphae of these two isolates also coiled around R. solani hyphae and the R. solani hyphae were cut in their presence. Penetration of R. solani hyphae by any of the Trichoderma spp. isolates was never observed by light microscopy.

Observations were made of hyphal interactions in addition to colony interactions (Table IV-1), for 11 Trichoderma spp. isolates and R. solani based on initially completed results of colony interactions. Table IV-1 shows that ten isolates (all except 85.12) grew over and sporulated on paired colonies of R. solani. Degree of fungal sporulation ranged from slight for three isolates, N35.5, 85.12.1 and 85.46.5a, to very intense for one isolate, N45.7. Mutual intermingling of hyphae was seen between all 11 isolates and R. solani. Hyphae of nine isolates (all 11 except 85.42.3a and 85.46.3a) showed coiling around hyphae of R. solani. Two of these isolates 85.12.2 and 85.46.5a,

exhibited an extensive amount of hyphal coiling around R. solani hyphae (figure IV-2 A). Rhizoctonia solani hyphae were cut into short segments in the presence of all 11 isolates, except 85.43.3a and 85.50.4a. The extent of cutting was very widespread in the presence of isolate 85.46.5a (figure IV-2 B). Only one of these isolates N45.7, had hyphae having swollen tips/appressoria when contacting R. solani hyphae.

In general, those Trichoderma spp. isolates exhibiting colony interactions of both overgrowth and sporulation on R. solani also resulted in hyphal interactions of coiling around and cutting of R. solani hyphae. Ten isolates, all except 85.12, overgrew and sporulated on R. solani. Seven of these ten, all except 85.43.3a, 85.46.3a and 85.50.4a, also resulted in both coiling around and cutting of R. solani hyphae (table IV-1).

Control of R. solani in soil by coating canola seed with Trichoderma spp.

In the study to determine the viability of Trichoderma spp. conidia coated onto Tobin seed it was found that colonies developed from all 14 coated Tobin seed for both isolates, N.45.7 and 85.12.2, after four days incubation of the seed on pda. This indicated that the conidia remained viable after having been collected from their cultures and applied to the canola seed.

Table IV-2 shows results of the experiment to control CSB by coating seed with either of two Trichoderma spp. isolates. This experiment was repeated once. Statistical analysis showed that the resulting ranking of treatments was the same each time the experiment was carried out.

In soil not infested with R. solani, emergence for the six treatments ranged from 95.2% for seed coated with either of the two Trichoderma spp. isolates, N45.7 and 85.12.2 (Tb-mcN, and Tb-mc85, respectively), to 84% for seed commercially treated with Vitavax fungicide (Tobin-V). Neither of the two Trichoderma spp. isolates significantly affected emergence compared to that of surface disinfected seed (Tb) or seed coated with methyl cellulose (Tb-mc). Emergence of Tobin-V was significantly lower

than Tb-mcN, Tb-mc85, Tb-mc or seed coated with the experimental fungicide mixture (Tb-ef). Disease-like symptoms were absent on all seedlings of Tb-mcN, Tb-mc, Tb-ef and Tobin-V. Symptoms were present on fewer than 2% of the seedlings of Tb or Tb-mc85.

Seedling % emergence was signficantly lower in R. solani-infested soil than in non-R. solani infested soil for each of the six seed treatments. In soil infested with R. solani, emergence ranged from 76.8% for Tb-ef to 14% for Tobin-V. Emergence of Tb-ef from R. solani-infested soil was statistically alike that of Tobin-V from non-R. solani infested soil and was significantly greater than that of all other seed treatments in R. solani-infested soil. Emergence of Tb-mcN and Tb-mc85 was not improved over that of Tb-mc or Tb. Seed commercially treated with Vitavax provided the lowest emergence, 14%, of all treatments from R. solani-infested soil. This emergence was similar to that provided by Tb-mcN and Tb-mc85. Canola seedling blight disease symptoms on seedlings ranged from 99.3% for Tb-ef to 100% for the other five treatments including the seed which was coated with the two Trichoderma spp. isolates (Tb-mcN and Tb-mc85).

Control of R. solani in soil by the use of Trichoderma sp.-wheat bran amendments

Microscopic observations of distilled water suspensions taken from the wb-Tr after ten day's incubation at rtl, showed the presence of a.) septate hyphae containing chlamydospores, and b.) many one-celled Trichoderma-like conidia, with many of the conidia having germinated and possessing long germ tubes. The wb-Tr was found to contain 4.6×10^8 viable propagules/g, based on completed colony counts on dilution plates incubated for two days at rtl. Surface disinfected Tobin seed used in this experiment, was found to have an 89% germination rate after 48 hours incubation.

Table IV-3 shows results of the experiment to control CSB by Trichoderma sp. amendments. This experiment was repeated once. Statistical analysis showed that the resulting ranking of treatments was similar each time the experiment was carried out.

In soil not infested with R. solani, Tobin emergence for the six treatments ranged from 76.0% for the wheat bran-Trichoderma sp. plus chitin (wbTrch) amendment to 54.0% for the wheat bran plus chitin (wbch) amendment. Neither the wheat bran-Trichoderma sp. (wbTr), or wbTrch amendments significantly affected emergence compared to that of seed alone. No seedlings had CSB-like symptoms from the seed alone, wbch or wbTrch amendments. Symptoms were present on 4.1% or less of the seedlings from the wbTr, chitin (ch) or wheat bran (wb) amendments.

In R. solani-infested soil, the wbTrch amendment resulted in a significant increase in emergence compared to that of Tobin seed alone (81.2 and 42.0% emergence, respectively). The wbTrch amendment also resulted in a significantly greater % emergence (81.2%), than the wbTr amendment (63.2%). The wbTr, wbch, and wb amendments resulted in emergence that was not significantly different from that of Tobin alone. The ch amendment resulted in a significantly reduced % emergence compared to that of seed alone. For all six treatments, % seedlings with CSB symptoms was significantly greater in R. solani-infested soil than in non-infested soil. In R. solani-infested soil 99% of the seedlings had CSB symptoms from the 'seed alone' treatment (Table IV-3). The ch, wbch and wb amendments did not significantly affect % of seedlings with symptoms, compared to that of Tobin seed alone. These amendments resulted 96.7 to 100% of the seedlings having symptoms. The wbTr and wbTrch amendments significantly reduced the % of seedings with CSB symptoms to 61.6 and 52.6%, respectively, compared to 99% for seed alone in R. solani-infested soil.

D. Discussion

Trichoderma spp. are widely distributed, occurring in most soils and natural habitats (15). In this study, Trichoderma spp. were found to be very widespread, being isolated from 44 of the 47 field and forest soils collected.

Observations of four types of colony interactions between 22 various Trichoderma spp. isolates and R. solani was one method used in the selection of possible antagonists to R. solani. The three most frequently observed colony interactions were mutual intermingling of hyphae, sporulation of the Trichoderma spp. isolates on R. solani and growth of the Trichoderma spp. isolates over R. solani. Growth of R. solani over the Trichoderma spp. isolates was the least frequently observed interaction. Mutual inhibition of colony growth, a type of colony interaction reported by Tsuneda (19), was not observed between any of the various Trichoderma spp. isolates and R. solani. These interactions were observed under a dissecting microscope at about 500x magnification. Based upon observations of this nature, it was sometimes difficult to discern which fungus was being observed. Generally however, R. solani hyphae could be identified because they were larger in diameter, with right angle branchings, compared to hyphae of the various Trichoderma spp. Very little differentiation could be made amongst the 22 isolates based on the studied colony interactions.

Observations of hyphal pairings, in this study, showed that most of the 24 Trichoderma spp. isolates had hyphae that coiled around R. solani hyphae and also resulted in cutting of R. solani hyphae into hyphal fragments. Intact hyphae of R. solani were always observed in the absence of any Trichoderma sp. isolate. Appressoriumlike structures were rarely seen on hyphae of any of the Trichoderma spp. isolates and penetration of R. solani hyphae by the Trichoderma spp. isolates was never observed. Hyphal interactions have been reported in other studies. Gliocladium catenulatum Gilman and Abbott, a hyperparasitic fungus of Fusarium spp. was shown to produce appressoriumlike structures and coiling hyphal branches on and around hyphae of

several Fusarium spp. (11). Hyphal interactions were also shown between Trichoderma hamatum and R. solani (5). These interactions included coiling and appressoriumlike structures of T. hamatum around and on R. solani and penetration and lysis of R. solani hyphae by T. hamatum.

A positive relationship was found between results of colony and hyphal interactions in pairing of R. solani and 11 Trichoderma spp. isolates. Nine of the 10 Trichoderma spp. isolates showing both sporulation on and growth over R. solani, also had hyphae coiling around and/or resulting in cutting of R. solani hyphae (Table IV-1).

Trichoderma harzianum 85.46.5a, underwent extensive colony (sporulation on and growth over R. solani) and hyphal (intense coiling around and cutting of R. solani) interactions with R. solani (Table IV-1) (figure IV-2). Trichoderma harzianum has been reported to parasitize R. solani by such means as coiling around R. solani hyphae and production of appressoriumlike structures (3,5,6).

Rhizoctonia solani caused damping-off has been reported to be controlled by coating seed with Trichoderma spp. Trichoderma hamatum applied as a seed coating to pea and radish seed reduced damping-off caused by R. solani and Pythium spp. (8). After two days incubation on moist filter paper, this antagonist was shown to have undergone extensive colonization and sporulation on coated radish seed. Trichoderma harzianum was unable to colonize pea seed after being applied to this seed and it did not control damping-off caused by Pythium spp. (8). Trichoderma spp. applied to seed differ in their ability to colonize the seed and also in their ability to control damping-off (8). Colonization of seed by Trichoderma spp. applied as seed coatings, may not be the same for seed on filter paper as in soil. Germination of Trichoderma spp. conidia applied to seed, may differ when seed is on filter paper or in soil. Natural field soil was shown to have an effect on germination of conidia and chlamydospores of three Trichoderma spp. (2). Germination of conidia of T. hamatum, T. harzianum and T. viride Pers. ex Fr. was reduced from a range of 15 to 57% on moist filter paper, to less than 22% (range of 1 to 22%) in

natural field soil. For these three species, germination of chlamydospores was greater than that of conidia on both moist filter paper and in natural soil. Chlamydospores had high levels of germination ranging from 67 to 92% on moist filter paper and 39 to 94% in natural field soil. The Trichoderma species involved, the ability of its spores to germinate and its ability to colonize seed are all factors involved in determining whether this fungus when applied to seed, can control damping-off caused by R. solani or Pythium spp..

Two Trichoderma spp. isolates N45.7 and 85.12.2, were chosen to be applied as seed coatings to control R. solani-caused CSB. Data was collected for percent emergence and percent seedlings with CSB symptoms which relate to the pre- and post-emergence damping-off phases of CSB. Both isolates underwent colony (intense sporulation on and growth over R. solani) and hyphal (coiling about and maceration of R. solani hyphae) interactions with R. solani (Table IV-1). Both the Trichoderma spp. isolates remained viable on coated seed. Colonies of both isolates developed from all coated seed placed on agar. Both Trichoderma spp. isolates had no deleterious effects on seedling emergence or on CSB symptoms on emerged seedlings compared to these for uncoated canola seed in soil not infested with R. solani (Table IV-2). Both Trichoderma sp. isolates, N.45.7 and 85.12.2 applied as seed coatings to cv. Tobin seed were ineffective in controlling CSB in R. solani-infested soil.

The Vitavax treated seed did not control R. solani-caused CSB. This seed had been stored in the laboratory for a long period of time possibly leading to the low level of seedling emergence (84%) and the ineffectiveness of this fungicide (Table IV-2). The experimental fungicide mixture (Benlate, Metalaxyl, and rizolex) provided for greatly improved seedling emergence but not reduced levels of seedlings with CSB symptoms compared to seed coated with the Trichoderma spp. and the uncoated seed.

The use of Trichoderma-colonized wheat bran amendments was considered for the control of R. solani caused CSB, because of the inability of the Trichoderma spp. seed

coatings to control this disease. Other studies have shown Trichoderma spp. colonized wheat bran amendments (T-wb) to be effective in controlling R. solani caused damping-off. (7,13). This study tested the effect of T. harzianum 85.46.5a, in T-wb amendments to control R. solani caused CSB. This isolate underwent extensive hyphal interactions (coiling around and cutting of R. solani hyphae) with R. solani on agar plates (Table IV-1) indicating its potential to control this pathogen in soil. The two T-wb amendments, with and without chitin, showed promise for the control of R. solani caused CSB (Table IV-3). In non-infested soil both these amendments did not significantly affect % emergence or % of seedlings with CSB symptoms, compared to that for seed alone. In R. solani-infested soil the T. harzianum-wb plus chitin amendment resulted in a significant increase in percent emergence and both wbTr and wbTrch amendments resulted in a significant decrease in % of seedlings with CSB symptoms compared to these for 'seed alone'. The addition of chitin (0.08 g/pot) in a layer over the seed in each pot significantly increased the effectiveness of T. harzianum 85.46.5a in controlling R. solani caused CSB. Seedling emergence for the T. harzianum-wb plus chitin amendment, was significantly greater than that for the T. harzianum-wb amendment (Table IV-3). It is known that Trichoderma spp. produce chitinase which degrades chitin (15). This production of chitinase by Trichoderma enables its degradation of fungi including R. solani containing chitin in their cell walls. Trichoderma harzianum was shown to excrete chitinase when it was grown in media containing chitin or cell walls of R. solani (4,17). Incorporation of chitin along with T. hamatum seed treatments was shown to result in a positive effect on control of seedling diseases caused by R. solani (9). In this study, the positive effect of chitin added to the T. harzianum-wb amendment and the significant reduction in both pre- and post-emergence damping-off of CSB, suggests that T. harzianum 85.46.5a, possibly produces chitinase involved in the control of R. solani and CSB. The addition of chitin to the T. harzianum-wheat bran amendment, may have

stimulated chitinase production by this T. harzianum isolate, thereby increasing the controlling effect of this amendment on R. solani caused CSB.

In soil not infested with R. solani, percent emergence for the 'seed alone' treatment in the T. harzianum-wheat bran amendment experiment was only 67.2% (Table IV-3). This compares to 90% emergence for the same treatment in the experiment with the Trichoderma spp. coated seed (Table IV-2). One possible explanation for the low emergence of 67.2% in the T. harzianum-wheat bran amendment experiment, was the relatively low level of seed germination which was 89% (on filter paper).

Table IV-1. Colony and hyphal interactions between R. solani and 11 Trichoderma spp. isolates

isolate[T]	Colony interactions			Hyphal interactions		
	MI	S	OT	CL	ST/A	CT
N.35.5	+	+	+	+	-	+
N.45.7	+	+ + +	+	+	+	+
85.12	+	-	-	+	-	+
85.12.1	+	+	+	+	-	+
85.12.2	+	+ +	+	+ +	-	+
85.20.3	+	+ +	+	+	-	+
85.43.3a	+	+ +	+	-	-	-
85.46.3a	+	+ +	+	-	-	+
85.46.5a	+	+	+	+ +	-	+ +
85.50.1a	+	+ +	+	+	-	+
85.50.4a	+	+ +	+	+	-	-

[T] 85.46.5a, T. harzianum

\+ presence of specific interactions*

\- absence of specific interactions

* degree of interaction indicated by numbers of +

MI- mutual intermingling hyphae

S- sporulation by Trichoderma sp. on R. solani

OT- overgrowth of Trichoderma sp. on R. solani

CL- coiling of hyphae of Trichoderma sp. on R. solani

ST/A- swollen tips/appressoria of Trichoderma sp. contacting R. solani

CT- maceration of hyphae of R. solani in presence of Trichoderma sp.

Table IV-2. Control of R. solani caused canola seedling blight, by coating seed with Trichoderma spp.

Treatment	R.s.#	% emergence[x]	% seedlings with CSB symptoms[o]
Tb-mcN	-	95.2 a	0.0 b
Tb-mc85	-	95.2 a	0.7 b
Tb-mc	-	94.8 a	0.0 b
Tb-ef	-	92.0 a	0.0 b
Tb	-	90.0 ab	1.6 b
Tobin-V	-	84.0 bc	0.0 b
Tb-ef	+	76.8 c	99.3 a
Tb-mc	+	28.0 d	100.0 a
Tb-mcN	+	22.8 de	100.0 a
Tb	+	21.2 de	100.0 a
Tb-mc85	+	14.0 e	100.0 a
Tobin-V	+	14.0 e	100.0 a

Treatments: Tb, Tobin seed; Tb-mc, Tobin with methyl cellulose; Tb-mcN, Tb-mc85, Tobin seed with methyl cellulose-Trichoderma sp. N.45.7, 85.12.2, respectively; Tb-ef, Tobin with experimental fungicide; Tobin-V, Tobin with Vitavax.

R.s.: +, R. solani-infested soil; -, absence of R. solani

x means based on 6 replicate pots; values with same letters are not significantly different at P=0.05 according to Duncan's multiple range test; letters from transformed data

o means based on only those replicate pots with emerged seedlings; values with same letters are not significantly different at P=0.05 according to Duncan's multiple range test; letters from transformed data

Table IV-3. Control of R. solani caused canola seedling blight, by Trichoderma sp. amendments

Treatment	R.s.#	% emergence[x]		% seedlings with CSB symptoms[o]	
wbTrch	+	81.2	a	52.6	b
wbTrch	-	76.0	ab	0.0	c
wb	-	69.2	abc	1.0	c
wbTr	-	69.2	abc	4.1	c
seed alone	-	67.2	abcd	0.0	c
ch	-	66.0	abcd	1.2	c
wbTr	+	63.2	bcd	61.6	b
wbch	-	54.0	cd	0.0	c
wbch	+	50.0	cd	100.0	a
wb	+	44.8	d	96.7	a
seed alone	+	42.0	d	99.0	a
ch	+	14.8	e	100.0	a

Treatments: seed alone, Tobin seed; wb, Tobin with wheat bran; ch, Tobin with chitin; wbch, Tobin with wheat bran and chitin; wbTr, Tobin with wheat bran-T. harzianum 85.46.5a; wbTrch, Tobin with wheat bran-T. harzianum 85.46.5a and chitin.

R.s.: +, R. solani-infested soil; -, absence of R. solani

x means based on 6 replicate pots; values with same letters are not significantly different at P=0.05 according to Duncan's multiple range test; letters from transformed data

o means based on only those replicate pots with emerged seedlings; values with same letters are not significantly different at P=0.05 according to Duncan's multiple range test; letters from transformed data

Figure IV-1. Map of Alberta showing locations of 47 sites from where soils were collected for isolation of Trichoderma spp.

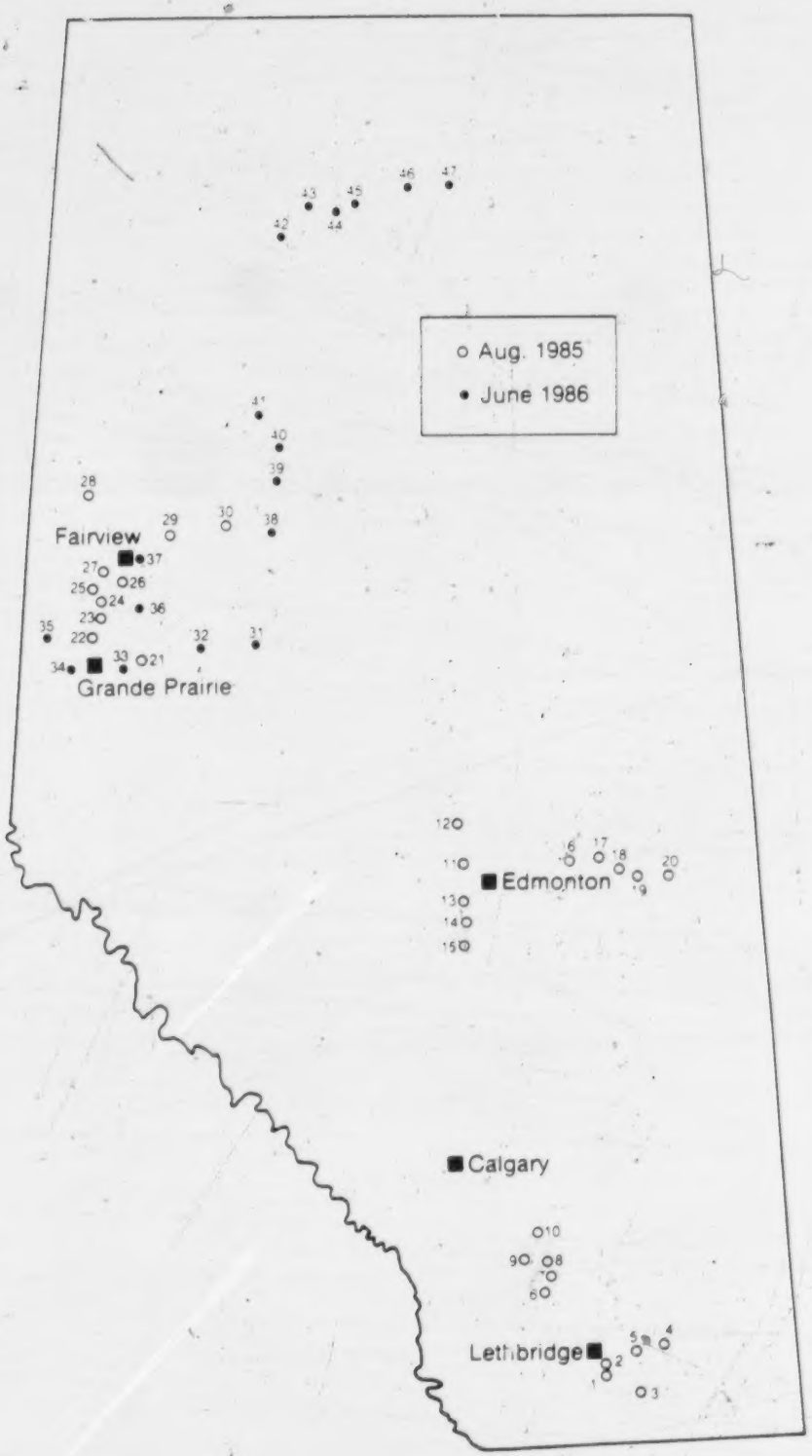
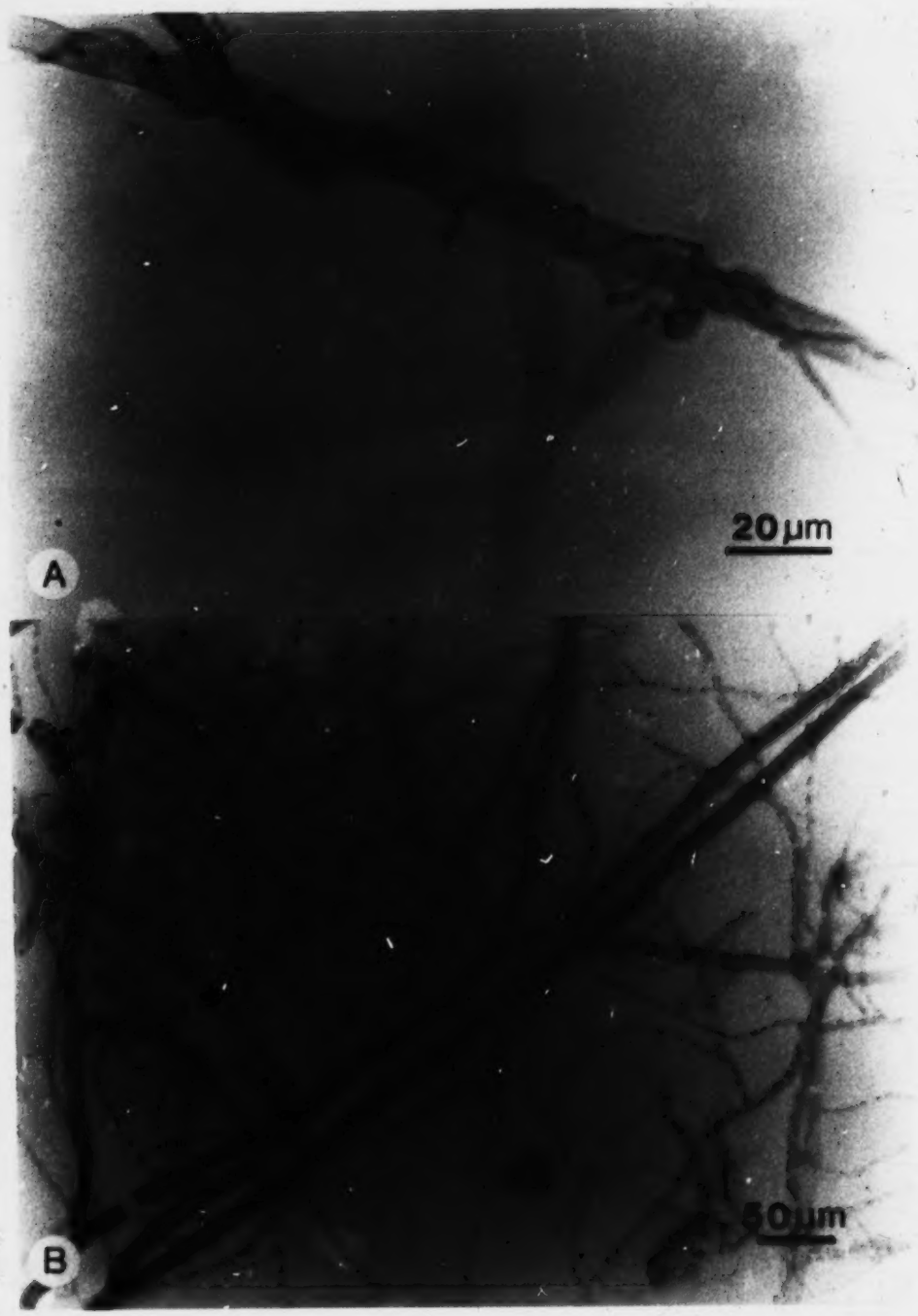

Aug. 1985
June 1986
Fairview
Grande Prairie
Edmonton
Calgary
Lethbridge

Figure IV-2. Hyphal interactions between Trichoderma harzianum 85.46.5a and Rhizoctonia solani.

(A). Trichoderma harzianum coiling around Rhizoctonia solani (x400).

(B). Rhizoctonia solani hyphae 'cut' into short segments in presence of Trichoderma harzianum (x160).


20 µm
A
B

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V. General discussion and conclusions

Canola seedling blight (CSB) was found to be widespread throughout Alberta with diseased seedlings being collected from 73 fields from southern Alberta around Lethbridge, to northern Alberta around Fairview and Grande Prairie. Rhizoctonia spp., Fusarium spp. and Pythium spp. accounted for 57.2% of the total numbers of fungi obtained from field seedlings with symptoms of this disease. Rhizoctonia spp. and Pythium spp. were the most and least numerous, respectively, of all identified fungi. The remaining 42.8% of the fungal isolates included some very abundant and frequently isolated saprophytes including Aspergillus spp., Penicillium spp. and Trichoderma spp. A recent study in Saskatchewan also found that Rhizoctonia spp., Fusarium spp. and Pythium spp. were the most to least frequently isolated fungi from canola seedlings with damping-off and root rot (12).

Fungi isolated from field seedlings with CSB symptoms included R. solani AG 2-1 and AG 4, Fusarium spp., F. acuminatum, F. avenaceum, P. paroecandrum and P. sylvaticum. Canola seed were colonized by P. paroecandrum, P. pythioides, P. rostratum, P. sylvaticum, P. ultimum, P. vanterpoolii and Pythium sp. Group G within three days of its being placed into various field soils. One question arising during the isolation of fungi from field seedlings was whether the Pythium spp. isolates representing 5.8% of the total fungi, colonized the seedlings after infection by Rhizoctonia spp. and/or Fusarium spp. The ability of Pythium spp. to colonize canola seed within three days of its being in field soil indicates that this fungus can act as an initial colonizer and pathogen causing CSB.

All the identified fungi from field seedlings and canola seed, except F. acuminatum, P. rostratum and P. vanterpoolii were pathogenic in causing CSB of B. campestris cvs. Candle or Tobin, resulting in significant reductions in % emergence and increases in % of seedlings with CSB symptoms. These symptoms were similar to those observed in the field seedlings. All tested R. solani isolates were highly virulent resulting in less than

20% emergence and 96.9-100% of the seedlings having disease symptoms. Rhizoctonia solani has been reported to be highly virulent to canola seedlings (3,5). Both F. avenaceum isolates were moderately virulent resulting in 65-69% emergence and 43-44% of the seedlings having CSB symptoms. Fusarium avenaceum has been reported to cause seedling death of rapeseed (1). Two earlier reports presented conflicting results about the pathogenicity of F. acuminatum to canola with its pathogenicity being confirmed to rapeseed (10) but not confirmed to canola (3). In this present study the F. acuminatum isolate at the tested inoculum level was non-pathogenic in causing CSB of Candle. This and any other F. acuminatum isolates should be retested at a higher inoculum level more similar to that of the two F. avenaceum isolates to confirm or deny its pathogenicity. Pythium paroecandrum, P. pythioides, P. sylvaticum and P. ultimum were found to be highly virulent resulting in less than 50% germination of seed, seedling emergence of about 50% or less and more than 50% of the seedlings having disease symptoms. Disease symptoms caused by R. solani, Fusarium sp., F. avenaceum and Pythium spp. included non-germinated seed being soft and mushy; hypocotyls being constricted, discolored (brown) and containing lesions of 1-3 mm length; and roots having discolored (brown) regions. Presence of dark brown dot lesions on cotyledons and upper portions of hypocotyls was characteristic of Pythium spp. infection. These dot lesions were absent on seedlings infected with R. solani or Fusarium spp.

Pathogenicity trials in this current study involved inocula of the same weight for the Fusarium spp. and R. solani isolates, or volume for the Pythium spp. isolates. All isolates produced different numbers of viable propagules and the inocula of these isolates contained different quantities of viable propagules. Analysis of pathogenicity data was based on this fact and involved comparisons being made between individual isolates and the control treatments. Further pathogenicity testing should involve inocula of the isolates of each genus containing the same quantity of viable propagules allowing for comparisons to be made amongst isolates of each genus.

Rhizoctonia solani the most important of the identified fungi causing CSB in Alberta, was isolated in the greatest numbers and was shown to be highly virulent in causing this disease.

Attempts were undertaken to control CSB in R. solani-infested soil with Trichoderma spp. Trichoderma spp. were obtained primarily from soil dilutions on agar media. No Trichoderma spp. were isolated from R. solani hyphae placed into soil, or from inhibition zones in R. solani colonies on agar media. Another technique for isolating Trichoderma spp. antagonistic to R. solani would involve collecting and culturing washings from roots and lower hypocotyls of canola field seedlings with CSB symptoms. Trichoderma spp. found to be effective in controlling grey mold caused by Botrytis spp. were isolated from foliar washings of bean plants (8). In the choosing of the most promising Trichoderma spp. to control CSB more emphasis was placed on the results from observations of specific hyphal interactions than from colony interactions. The two Trichoderma spp. isolates applied as seed coatings to Tobin were ineffective in controlling CSB in R. solani-infested soil. The experimental fungicide mixture containing Benlate, Metalaxyl and rizolex was effective in improving only % emergence. Rizolex has been reported to be effective against R. solani (previously mentioned).

Trichoderma harzianum applied as a wheat bran-chitin amendment was effective in controlling CSB of B. campestris cv. Tobin in a R. solani-infested soil, resulting in a significant increase in % emergence and reduction in % of seedlings with CSB symptoms. Trichoderma harzianum applied as a wheat bran amendment resulted in a significant reduction in % of seedlings with CSB symptoms. Trichoderma spp. including T. harzianum applied as wheat bran amendments have successfully controlled a number of pathogens including R. solani and seedling diseases, with chitin being shown to stimulate chitinase production by Trichoderma spp. (2,4,7,9). The wheat bran-chitin amendment had two potential beneficial effects on T. harzianum with the wheat bran acting as a food base and the chitin stimulating possible production of chitinase by T. harzianum.

A. Suggestions for Future Studies

Future research based on results from these studies should examine:

1.) Identification and pathogenicity testing of any additional Fusarium spp. and Pythium spp. isolated from field seedlings and seed.

2.) Control of CSB by T. harzianum applied onto seed or as wheat bran/alginate amendments: mechanisms involved in control of R. solani by T. harzianum; effects of various amounts of conidia applied to seed and the application of chlamydospores rather than conidia onto seed or into amendments (6,11); supplementary compounds including chitin added to treatments or amendments; and field testing of effective treatments.

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